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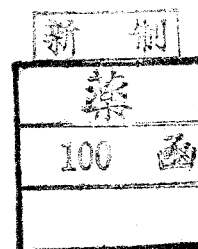
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TOTAL SYNTHESIS OF BOVINE PANCREATIC RIBONUCLEASE A

1980

Nobutaka Fujii

TOTAL SYNTHESIS OF BOVINE PANCREATIC RIBONUCLEASE A

Thesis Presented for

The Degree of Doctor of Pharmaceutical Sciences

Nobutaka Fujii

Faculty of Pharmaceutical Sciences
Kyoto University

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Chapter 1. Introduction

Early in this century, Emil Fischer¹⁾ found that proteins are composed of amino acids through studies on "peptone" formed by their partial hydrolysis and dreamed to synthesize biologically active proteins starting with amino acids by means of organic chemistry. His fundamental contributions, such as an introduction of an idea of the urethane-type protecting group, founded the development of present peptide chemistry. Ever since, many organic chemists engaged in peptide synthesis, but no really significant developments were achieved for about two decades. It was not until 1932 that the unsurpassed discovery of the catalytically removable protecting group, the benzyloxycarbonyl(Z) group of M. Bergmann and L. Zervas,²⁾ led to revolutionary developments in this field. As a consequence of this great contribution, syntheses of naturally occurring small peptides, such as carnosine³⁾ and glutathione,⁴⁾ were soon achieved and thus the Fischer's dream got one step closed to reality.

In 1953, the structure of a pituitary hormone, oxytocin, was elucidated by du Vigneaud and his associates⁵⁾ and its synthesis was simultaneously accomplished by the same group, using Na-*liq.* NH₃ as the deprotecting procedure.⁶⁾ The Fischer's dream was thus realized for the first time in the field of peptide hormone. Since then, a number of peptide hormones have been synthesized. Syntheses of insulin⁷⁾ and adrenocorticotropins,⁸⁾ for example, are the another successful achievements in this field. However, synthesis of fully active enzymes, representative proteins in nature, has remained to be accomplished to date, despite numerous efforts of organic chemists. For example, solid phase syntheses of ribonuclease A by Bittman and Merrifield,⁹⁾ hen egg white lysozyme by Sharp et al.,¹⁰⁾ and ribonuclease T₁ by Izumiya et al.¹¹⁾ produced only partial enzymic

activities respectively. None of the synthesis of enzymes in a conventional manner, such as ribonuclease T₁ by Hofmann et al.¹²⁾, lysozyme analog by Galpin et al.¹³⁾, iso-1-cytochrome C by Moroder et al.¹⁴⁾ and Δ^5 -3-ketosteroid isomerase by Kisfaludy et al.¹⁵⁾ has still be accomplished

This thesis describes the first synthesis of a protein with the full enzymic activity of bovine pancreatic ribonuclease (RNase A.¹⁶, *foot note)

Before entering to the main subject, the author wishes to discuss briefly the general idea for this protein synthesis. The principle of peptide synthesis is composed of three elements; (1) amide bond formation, (2) suppression of racemization, and (3) choice of protecting groups and deprotection. Considering various methodologies employed in the previous attempts for protein syntheses mentioned above, the present synthesis of RNase A was conducted along the following principles: (1) The peptide bond was constructed in the conventional manner, instead of the solid phase method.¹⁷⁾ Since in the latter method, removal of delineated peptides produced by incomplete condensations of amino acids on a resin is practically impossible and consequently the possibility is great to afford impure peptides. (2) Useful informations are now available for suppression of racemization during the bond formation. However a degree of racemization suppressed by addition of N-hydroxysuccinimide¹⁸⁾ or 1-hydroxybenzotriazole¹⁹⁾ during the dicyclohexylcarbodiimide condensation is still not superior to that of the azide procedure.²⁰⁾ Therefore the Rudinger's azide procedure²¹⁾ was selected as a main tool for construction of the entire peptide backbone. (3) In this synthetic studies, the problem to which the author concerned mostly, was the device of a new deprotecting procedure, since desired peptides can not be obtained without selection of a suitable deprotecting procedure, even if the peptide bond is far elongated. In order to remove steadily so many protecting groups required for synthesis of

complex proteins, methanesulphonic acid was introduced as a de-protecting reagent²²⁾ and this new reagent was applied to the present synthesis of RNase A.

* *Following mammalian RNase structures have been elucidated.*

Porcine; R.L. Jackson and C.H.W. Hirs, J. Biol. Chem., **245**, 637 (1970): Sheep; R. Kobayashi, C.H.W. Hirs, and M. Hagenhuber, J. Biol. Chem., **248**, 7833 (1973): Rat; J.J. Beintema and M. Gruber, Biochim. Biophys. Acta, **310**, 161 (1973): Horse; A.J. Scheffer and J.J. Beintema, Eur. J. Biochem., **46**, 221 (1974): Goat; G.W. Welling, A.J. Scheffer, and J.J. Beintema, FEBS Lett., **41**, 58 (1974): Girafe; W. Gaastra, G. Groen, G.W. Welling, and J.J. Beintema, FEBS Lett., **41**, 227 (1974): GNU; G. Groen, G.W. Welling, and J.J. Beintema, FEBS Lett., **60**, 300 (1975): Reindeer; G.L. van den Berg and J.J. Beintema, FEBS Lett., **56**, 101 (1975): Topi; H. Kupper and J.J. Beintema, Biochim. Biophys. Acta, **446**, 337 (1976): Coypu and Chinchilla; A. van den Berg, L. van den Hende-Timmer, and J.J. Beintema, Biochim. Biophys. Acta, **453**, 400 (1976): Whale; G.W. Welling and J.J. Beintema, Biochem. J., **157**, 317 (1976): Camel; G.W. Welling, H. Mulder, and J.J. Beintema, Biochem. Genet., **14**, 309 (1976): Muskrat; H.V. Dijk, B. Sloots, A. van den Berg, W. Gaastra, and J.J. Beintema, Int. J. Pept. Protein Res., **8**, 305 (1976): Kangaroo; W. Gaastra, G.W. Welling, and J.J. Beintema, Eur. J. Biochem., **86**, 2009 (1978): Mouse; J.A. Lenstra and J.J. Beintema, Eur. J. Biochem., **98**, 399 (1979): Hamster; P.A. Jekel, H.J. Sips, and J.J. Beintema, Biochimie, **61**, 751 (1979).

Amino acids, peptides and their derivatives mentioned in this thesis are of the L-configuration. Abbreviations used are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature [Biochem. J., 102, 23 (1967), 104, 17 (1967), 126, 773 (1972)].

Amino protecting groups:

- Z = benzyloxycarbonyl
- Z(OMe) = p-methoxybenzyloxycarbonyl
- Boc = tert-butoxycarbonyl
- NPS = o-nitrophenylsulphenyl
- For = formyl

Carboxyl protecting groups:

- Bzl = benzyl
- Bu^t = tert-butyl

Side chain protecting groups:

- Tos = p-toluenesulphonyl
- MBS = p-methoxybenzenesulphonyl
- MBzl = p-methoxybenzyl

Active esters and related derivatives:

- NP = p-nitrophenyl
- DNP = 2,4-dinitrophenyl
- Su = N-hydroxysuccinyl
- PCP = pentachlorophenyl
- TCP = 2,4,5-trichlorophenyl
- QCl = 5-chloro-8-quinolyl
- Troc = β,β,β -trichloroethyloxycarbonyl
- Tri = trityl
- Picoc = 4-picolyloxycarbonyl

Coupling reagents:

- DCC = dicyclohexylcarbodiimide
- IIDQ = 1-isobutoxycarbonyl, 2-isobutoxy-1,2-dihydroquinoline
- HOBt = 1-hydroxybenzotriazole

The following abbreviations are used for solvents and other reagents:

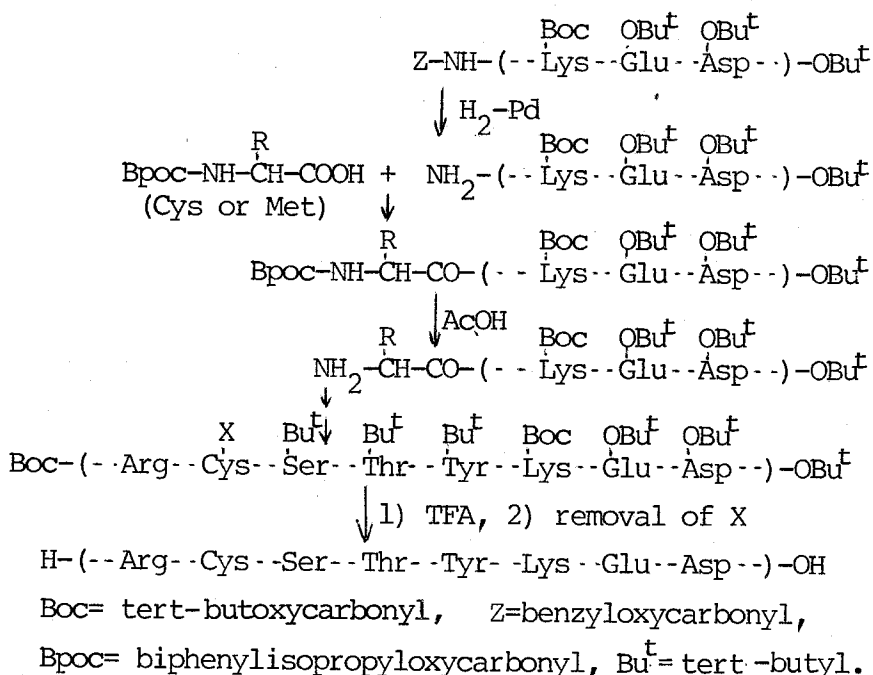
DMF	= dimethylformamide
THF	= tetrahydrofuran
DMSO	= dimethylsulphoxide
NMP	= N-methylpyrrolidone
HMPA	= hexamethylphosphoramide
TFA	= trifluoroacetic acid
EDTA	= ethylenediaminetetraacetic acid disodium salt

Chapter 2. Examination of Deprotecting Reagents for Peptide Sythesis

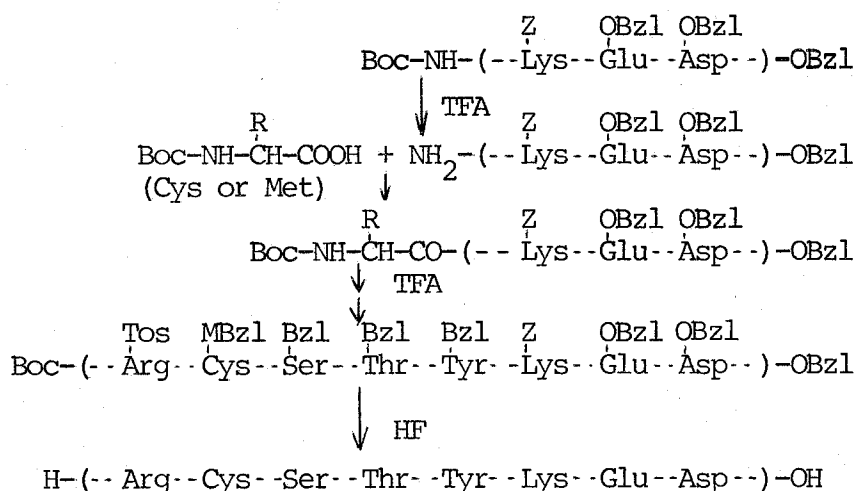
Section 1. Trifluoromethanesulphonic Acid, as a Deprotecting Reagent in Peptide Chemistry

The progress of peptide synthesis depends, in the author's opinion, mostly on the progress of deprotecting procedures. The catalytic hydrogenation procedure for the Z group²⁾ can be applied to the synthesis of peptides containing no cysteine or methionine and, therefore, du Vigneaud's synthesis of oxytocin⁶⁾ containing cysteine residues was accomplished by the Na-liq. NH₃ procedure. After recognizing an undesired side reaction, i.e., the cleavage of proline bond,²³⁾ in the Na-liq. NH₃ procedure, the present peptide syntheses are currently conducted by the following two representative procedures,

- (1) The trifluoroacetic acid procedure²⁴⁾ for removal of protecting groups based on tert-butylalcohol,



(2) The HF procedure²⁵⁾ for removal of protecting groups based on benzyl alcohol.



Bzl= benzyl, MBzl= p-methoxybenzyl, Tos= tosyl.

In the former procedure, three amino protecting groups in total, Z, Boc and Bpoc, are required for the synthesis of proteins containing lysine, cysteine and methionine and no suitable protecting groups for arginine removable by TFA are available at the present time. Because of these difficulties, such a procedure was not applied for the present synthesis.

As far as the choice of protecting groups is concerned, the principle of the latter synthesis is equivalent to that of the solid phase procedure.¹⁷⁾ Protected peptide can be synthesized by combination of the Boc or Z(OMe) group as an α -amino protecting group with the benzyl-type protecting groups as the side chain protection, regardless of presence or absence of sulphur containing amino acids. From the viewpoint of construction of peptide backbone, the HF procedure has an advantageous feature, compared to the TFA procedure. Despite such advantage, HF gives occasionally the Friedel-Crafts type side reaction and the reaction has to be performed in a special equipment. Therefore, it was decided to investigate alternative or better deprotecting reagents than HF. After surveying the acidi-

ty of various organic acids available as reagents, the author examined behaviours of protecting groups toward the action of organic sulphonic acids, namely trifluoromethanesulphonic acid²⁶⁾ and methanesulphonic acid²²⁾ as described in the following sections. Validity of these reagents to practical peptide synthesis was next examined.

First the capability of trifluoromethanesulphonic acid (TFMSA) as a deprotecting reagent was examined. TFMSA is a strong organic acid and its relative acidity to that of HCl is 47.²⁷⁾ As expected, TFMSA is acidic enough to cleave the Z group within 15 min at room temperature. It was found also that this reagent removed various protecting groups currently employed in peptide chemistry (Table).

Each amino acid derivative was dissolved at 20° in methylene chloride or TFA, and 5-10 equivalents of the reagent were employed. In order to suppress the possible alkylation reaction, anisole (1.5-3 equivalents) was used as a scavenger. A part of the solution was subjected to quantitative amino acid analysis.

Acid labile amino-protecting groups, such as Boc, Z(OMe) and NPS, were cleaved quantitatively within 3-5 min. In addition to the tert-butyl ester group, the benzyl group attached at the ω -carboxyl function of Glu and Asp and at the hydroxyl function of Ser and Thr was removed completely within 30 min. Treatment of Z-Trp-OH and Z-Tyr-OH with this reagent for 30 min regenerated the parent amino acids in nearly quantitative yields. However, the recovery of Tyr, after similar treatment of H-Tyr(Bzl)-OH, was low because of the predominant formation of the rearrangement product, 3-benzyl-tyrosine, which is known as the side product during the HF treatment²⁸⁾ also. Under similar conditions, recovery of Met from Z-Met-OH was also low. However, some improvement was achieved, when the treatment was performed at 0° in the presence of anisole plus methyl ethyl sulphide or dithiothreitol. This unexpected phenomenon

Table Removal of various protecting groups by TFMSA.

Treated amino- acid derivatives	parent amino acid regenerated(%)			
	3-5 min	15 min	30 min	60 min
NPS-Val-OH (a)	99.9			
Boc-Ser-OH (a)	99.2			
Z(OMe)-Gly-OH (a)	99.8			
Z-Trp-OH	85.6	94.6	100.9	
Z-Tyr-OH		92.4		
Z-Met-OH (b)		48.1	88.3	
Z-Glu-OH ^t	91.7	100.1		
Z-Glu(OBu)-OH		98.8		
H-Glu(OBzl)-OH	98.8	100.7		
H-Asp(OBzl)-OH	95.8	97.2	100.4	
H-Ser(Bzl)-OH	76.3	96.3	100.4	
H-Thr(Bzl)-OH	91.7	96.5	99.0	
H-Tyr(Bzl)-OH		10.7	10.5	
H-Arg(Tos)-OH (c)	85.8	87.1	93.3	100.0
H-Arg(NO ₂)-OH		21.3	33.5	34.5
H-His(Tos)-OH	77.9	79.1	84.3	98.4
H-Cys(Bzl)-OH (c)		62.0	98.7	
H-Cys(MBzl)-OH	99.8	100.0		

(a) methylene chloride was employed as a solvent.

(b) reaction in the presence of dithiothreitol.

(c) reaction at 40°.

will be discussed in Section 3.

Regeneration of Arg from H-Arg(Tos)-OH was achieved quantitatively after treatment with this reagent at 40° for 60 min, but only 35% was regenerated from H-Arg(NO₂)-OH under identical conditions. In this model experiment, the S-p-methoxybenzyl group of Cys was found to be cleaved within 15 min, but removal of the S-benzyl group required somewhat elevated temperature (40°).

From the result so far obtained, TFMSA seems to have a similar action to HF.

Section 2. Methanesulphonic Acid, as a Deprotecting Reagent in Peptide Chemistry

Succeeding to the TFMSA deprotection, the author examined the similar acidolysis of various protecting groups by methanesulphonic acid (MSA),²²⁾ together with a few other sulphonic acids for comparison. MSA is a weaker acid than TFMSA and its relative acidity to that of HCl is 1.9.²⁷⁾

In the presence of anisole, as a scavenger, each amino acid derivative was treated with MSA at room temperature for 30 min, and a part of the solution was subjected to quantitative amino acid analysis, as in the former experiments.

MSA cleaved also most of the protecting groups listed in Table, but was less effective in cases of H-Arg(NO₂)-OH, H-Arg(Tos)-OH, H-His(Tos)-OH and H-Cys(Bzl)-OH as compared to the results of TFMSA. Low recovery of Tyr was also due to the formation of 3-benzyltyrosine. Recovery of Met was also low, when this treatment was performed without addition of thiols. These experimental results suggested that if some considerations were taken especially to Met and Arg, MSA could be used like HF, because this acid, though it is a weaker acid than TFMSA, had still the enough ability to cleave the benzyl-type protecting groups, such as Z and Bzl ester.

At this convenience, the behaviour of Z group toward the action of 3-bromocamphor-8 or 10-sulphonic acid was examined. Regeneration of Glu from Z-Glu-OH was 85.9 or 82% respectively, after similar treatment with one of these reagents in TFA at room temperature for 60 min. However, these reagents were judged not to give any superior result, compared to MSA.

These two parallel experimental results described above now opened the possibility that both reagents, TFMSA and MSA, can be used along the similar principle as HF. Next, the author examined

Table Removal of various protecting groups by MSA
(20°, 30 min)

Treated amino acid derivatives	Parent amino acid regenerated (%)
NPS-Val-OH	100.5
Boc-Ser-OH	96.5
Z(OMe)-Gly-OH	94.0
Z-Trp-OH	100.0
Z-Tyr-OH	100.1
Z-Met-OH	31.5
Z-Glu-OH	101.2
Z-Glu(OBu ^t)-OH	99.5
H-Glu(OBzl)-OH	99.6
H-Asp(OBzl)-OH	100.8
H-Ser(Bzl)-OH	97.7
H-Thr(Bzl)-OH	101.6
H-Tyr(Bzl)-OH	30.2
H-Arg(NO ₂)-OH	58.5
H-Arg(Tos)-OH	49.2
Z-Arg(Z ₂)-OH	100.0
Z(OMe)-His(Tos)-OH	27.8
H-His(Bzl)-OH	0
H-Cys(Bzl)-OH	32.9
H-Cys(MBzl)-OH	94.6
H-Lys(For)-OH	0

various problems which may be arise, when these reagents are applied to practical peptide synthesis. It seems noteworthy that Glu was regenerated quantitatively from Z-Glu(OBzl)-OH by these reagents, indicating that these reagents do not give the Friedel-Crafts type side reaction, while treatment of Glu(OBzl) with HF in the presence of anisole is known to give a side product, γ -anisylglutamic acid.²⁹⁾ Low recovery of Tyr from Tyr(Bzl) indicated that peptides containing Tyr can well be synthesized, when unprotected Tyr is employed, instead of Tyr(Bzl). Considering the N^G-protection of Arg, a more acid labile protecting group than Tos is desirable. In 1976, Nishimura and Fujino³⁰⁾ introduced Arg(MBS). This protecting group was proven to be cleaved by TFMSA, as well as MSA, at room temperature within 60 min and this paved the way of synthe-

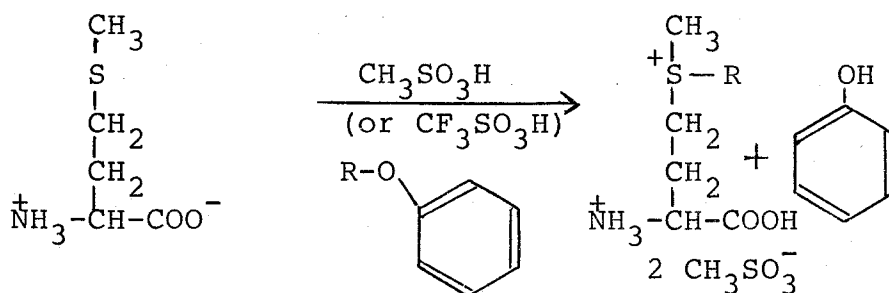
sizing peptides containing Arg by applying these reagents. From these considerations, the author has come nearly to the conclusion that if the unknown side reaction at Met is solved, these reagents will obtain qualification as a practical reagent in peptide synthesis. This pending problem will be discussed in Section 3.

Section 3. Examination of Methanesulphonic Acid Procedure for the Synthesis of Peptides containing Methionine

As described in the former sections, it was found that, when exposed to TFMSA or MSA, recovery of Met was very poor, because of the undesirable formation of presumably the same by-product. Each product was found detectable at the identical position on the short column of an amino acid analyzer, eluted between His and ammonia. Recovery of Met was improved to a certain extent by addition of sulphur compounds, but not satisfactorily enough.

This side product was now isolated for identification.³¹⁾ When a mixture of Met and anisole in MSA was kept at room temperature for 24 hr, this by-product was isolated in quantitative yield as the sole product, recrystallizable from alcohol. The structure of this compound was assigned as S-methylmethionine dimethanesulphonate (I) from infrared (IR), ^1H and ^{13}C nuclear magnetic resonance (NMR) spectral data. Especially, in the ^1H NMR (D_2O) spectra, two singlet peaks (δ 2.80 and 2.97, each corresponds to 6H of two methyl groups) were observed, indicating the presence of S-methyl sulphonium moiety in (I). Though the mass spectral data were not available, because of its non-volatility, the elemental analysis matched well the assigned formula. This structural assignment was then confirmed by direct comparison with the authentic sample of (I), derived from S-methylmethionine iodide³²⁾ by treatment of MSA via the corresponding dipicrate. Next, Met was similarly treated with 33% TFMSA-TFA in the presence of anisole. It was found that the sole product thus formed could be converted into the dipicrate in the same manner as the iodide mentioned above.

These experimental results suggested strongly that the sulphur atom of Met was trapped the methyl group of anisole as a cation. In order to confirm further this most plausible origin of the



methyl group in (I), phenetole, instead of anisole, was exposed to MSA in the presence of Met. Here, S-ethylmethionine sulphonium salt (II) was isolated as shown in the above Fig. It should be mentioned that acidic cleavage of anisole or phenetole did not take place, when Met was absent, and, in addition, Met in these acids gave no observable side reaction in the absence of anisole or phenetole. Thus, the role of Met became evident for the facilitated cleavage of aromatic ethers by these acids. Transfer of the methyl group from anisole to the sulphur atom of Met can be explained by the Hard-Soft concept,³³⁾ i.e., CH₃⁺ (soft acid) has an appreciable affinity with sulphur (soft base). This hitherto unknown reaction may be applied to the hydrolytic cleavage of aromatic ethers, since anisole gave phenol with MSA-Met at room temperature in reasonable yield. This new reaction was successfully applied to the regioselective cleavage of aromatic methyl ether of 4,5-dimethoxyindan-1-one.³⁴⁾

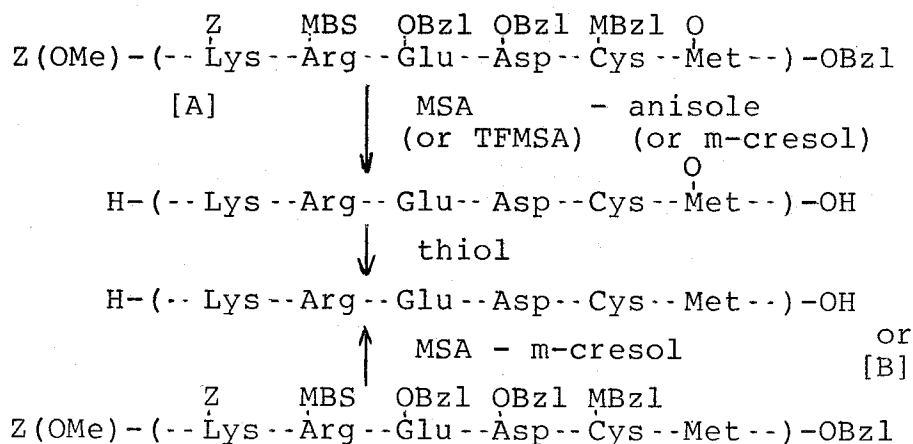
In connection with these observations, it is known that the sulphur atom of Met has a great tendency to be alkylated with the benzyl or tert-butyl cation liberated from the protecting groups. When the Z group was removed from Met-containing peptides acidolytically by hydrogen bromide, formation of the S-benzyl sulphonium salt was reported by Guttman and Boissonnas.³⁵⁾ More recently, Noble et al.³⁶⁾ isolated a tert-butyl sulphonium form of Met-containing pep-

tide after treatment of Boc-peptide with HF, even in the presence of anisole.

From the above experimental results and references, it can be judged that the thioether moiety of Met had better be protected, when the Z and Boc groups, possibly the benzyl and tert-butyl esters, are cleaved acidolytically with hydrogen bromide or HF. When TFMSA-anisole or MSA-anisole is applied as a deprotecting reagent, its protection is absolutely necessary. For this requirement, the sulfoxide, Met(O), seems to be the only compound available in the present peptide synthesis. The use of this compound was first recommended by Iselin³⁷⁾ because of its reversible conversion to Met with thiols. Through this protection, it is possible to prevent not only the partial alkylation of the parent amino acid at the deprotecting stage, but also the partial oxidation during the peptide synthesis. It was first confirmed that Met(O) is stable to TFMSA and MSA under deprotecting conditions. Thus, Met-containing peptides could be synthesized in such a manner that protected Met(O)-peptides are exposed to TFMSA-anisole or MSA-anisole and then the products are incubated with thiols to reduce Met(O) to Met. Alternatively, as seen from the reaction mechanism described above, Met-containing peptides can be synthesized starting with unprotected Met or by reduction of Met(O)-peptides with thiols prior to deprotection with these acids, if anisole is replaced by another scavenger, such as cresol, which has no property as an alkyl donor.

Here, the author could accumulate most of informations necessary to apply these two reagents, TFMSA and MSA, to practical peptide synthesis. Considering the acidities of these two reagents and other informations as described in the latter Chapter, the author reached the conclusion that complex peptides can be synthesized using MSA as a deprotecting reagent according to either one, [A] or [B], of the following two schemes. In the route [A], anisole can be applied as a cation scavenger by the reason stated above and Met(O)

is reduced after deprotection. In the route [B], Met(O) is reduced prior to deprotection and, therefore, anisole should be replaced by another scavenger, such as m-cresol for the MSA treatment. As will be discussed in Chapter 3, Section 6, synthesis of RNase A had to be performed by the route [B], because of the unexpected formation of sulphoxide of Cys(MBzl).³⁸⁾



As far as acidolytic deprotecting procedures are concerned, every deprotecting reagent, HF or TFA, as well as TFMSA or MSA, offers more or less common problems as regards to the necessity for suppression of side reactions due to alkyl cations generated from protecting groups. It is true that suitable scavengers play an important role for this purpose. Though m-cresol was selected as a cation scavenger, these accompanying problems will be again discussed in appropriate places in the next main Chapter.

Chapter 3. Total Synthesis of Bovine Pancreatic Ribonuclease A

Section 1. Synthesis of the Protected Pentadecapeptide Ester (Positions 110-124)

Summary

Main strategy for the total synthesis of bovine pancreatic ribonuclease (RNase) A is outlined with respect to deprotecting procedure and the selection of methods for construction of the entire amino acid sequence of this enzyme. Amino acid derivatives bearing protecting groups removable by methanesulphonic acid were employed in combination with the TFA labile N^{α} -protecting group, Z(OMe). Relatively small 30 peptide fragments were selected as the building blocks for construction of the entire peptide backbone. As Section 1, the C-terminal protected pentadecapeptide ester, Z(OMe)-(RNase 110-124)-OBzl, was synthesized by successive condensations of three fragments, Z(OMe)-Val-His-Phe-Asp(OBzl)-Ala-Ser-Val-OBzl [1], Z(OMe)-Asn-Pro-Tyr-Val-Pro-OH [2] and Z(OMe)-Cys(MBzl)-Glu(OBzl)-Gly-OH [3].

In this chapter, the author describes the results of studies on the total chemical synthesis of a protein with full and specific ribonuclease (RNase) A activity.

The primary structure of bovine pancreatic ribonuclease A was disclosed by Hirs, Moore and Stein³⁹⁾ in 1960. Shortly after, with the aid of improved methods for sequential analysis, the entire amino acid sequence consisting of 124 amino acids was confirmed¹⁶⁾ with minor revisions.⁴⁰⁾ Thus this protein became the first enzyme, for which the entire structure was recorded in literature. These authors emphasized that "a formula derived by degradative experiments could be regarded as a working hypothesis until it is confirmed by other means, preferably by chemical synthesis."

In 1969, two groups of investigators reported the chemical synthesis of materials with partial enzyme activity of RNase.^{9,41)} Gutte and Merrifield,⁹⁾ using the automated solid phase synthesis, reported that they succeeded in the synthesis of 0.41 mg of a protein (in the supernatant solution of ammonium sulphate fractionation of the trypsin-resistant material) with a specific activity against RNA of 78%. Various side reactions that could occur during the HF treatment²⁵⁾ of protected peptides were not clear at that time; for instances, γ -anisylation of Glu(OBzl)²⁹⁾ and the intramolecular rearrangement of Tyr(Bzl) to Tyr(3-Bzl).²⁸⁾ The latter became an enthusiastic subject of investigations by Merrifield and his associates⁴²⁾ in subsequent years. Accumulation of errors or failure sequences are often observed in the stepwise solid phase synthesis as reported by Tregear et al.⁴³⁾ using Edman procedure. As pointed out by Jones and Ridge,⁴⁴⁾ if the synthesis is carried out without purification and characterization of the intermediates, the presence of active molecules closely related to, but not identical with, the natural enzyme can not be excluded.

The other group, Denkwalter et al.,⁴¹⁾ reported the synthesis of S-protein (tetrahepta peptide, RNase 21-124)⁴⁵⁾ in a conventional

manner using a modified Leuch's anhydride procedure⁴⁶⁾ as the main strategy for the preparation of necessary fragments with minimal protection. After the Rudinger's azide condensation²¹⁾ of two large fragments, (position 21-64) and (position 65-124), the Z group from lysine and the newly devised acetamidomethyl group⁴⁷⁾ from cysteine were removed by HF and mercuric acetate respectively. They obtained a solution containing ca. 2% of S'-activity, upon combination of S-peptide (RNase 1-20) derived from the natural source. Thus the synthetic material was estimated to have an activity of 30-40% of the native enzyme, but this minute amount made further purification and characterization of the end product impossible. Thus, the author became aware that many problems remained to be solved and unambiguous synthesis of RNase A or S-protein remained to be accomplished.

The present synthesis of a protein with 124 amino acids corresponding to the entire amino acid sequence of RNase A was accomplished in a conventional manner by successive assembling of 30 peptide fragments, each of which was fully characterized by elemental and amino acid analyses and, after each condensation, all protected intermediates were also characterized. In the final step of the synthesis, methanesulphonic acid (MSA)²²⁾ was employed as a deprotecting reagent. After air oxidation,⁴⁸⁾ the crude material (9-12% activity) was purified by affinity chromatography⁴⁹⁾ to a more active compound (74-82% activity) followed by ion-exchange chromatography to essentially the fully active component. The synthetic enzyme, thus obtained, exhibited identical behaviour to natural RNase A upon electrophoresis and specific activity against yeast RNA and 2', 3'-cyclic cytidine phosphate.

The synthetic strategy, outlined above, is written schematically in Fig. 1. In order to construct the peptide backbone, a conventional procedure which would permit the characterization of each intermediate at each step was selected. In order to facilitate the

purification task, relatively small peptide fragments were selected as building blocks and these were assembled for the most part using a modified azide procedure.²¹⁾ These building blocks were condensed stepwise from C-terminal toward the N-terminal end. This approach requires more coupling steps compared to the condensation of relatively large fragments, but retains the advantageous feature of ease of purification of the protected intermediates. Smaller peptides couple efficiently by the azide procedure; however the efficiency decreased in a certain degree when the amino component reached around the S-protein stage. After reactions, the excess azide component could in most cases be readily removed by repeated precipitation. Although chromatographic techniques are usually of limited value in purifying large protected peptides, it was found that, in certain instances, the Sephacryl S-200 -5% H₂O-NMP (or DMSO) system is quite effective and preferable to Sephadex LH-20 or 60. Accordingly this technique was applied as certain steps of the RNase synthesis.

The methanesulphonic acid (MSA) deprotecting procedure²²⁾ employed in this synthesis was first introduced as described (Chapter 2) in 1975 and its scope and limitation have been examined through syntheses of model peptides, such as duck glucagon⁵⁰⁾ and human β -endorphin.⁵¹⁾ To remove, by a single treatment at the final step of the synthesis, the many protecting groups necessary to construct the peptide chain, the author considered the use of a strong acid, as for example, trifluoromethanesulphonic acid (TFMSA)-TFA.²⁶⁾ However, this acid was found to decompose Cys(MBzl)⁵²⁾ and to give Lys(Bzl) from Lys(Z). The latter side reaction, pointed out by Mitchell and Merrifield,⁵³⁾ did not occur in the MSA deprotecting system⁵⁴⁾ and, in this weaker acid system, full recovery of cysteine from Cys(MBzl) was obtained. It was further found that this acid has no tendency to form a γ -anisyl-glutamic acid derivative²⁹⁾ from Glu-(OBzl), a Friedel-Crafts type reaction known as a side reaction of

HF. Thus MSA was judged to be more attractive than either TFMSA or HF.

However, there were two additional problems to be solved before applying this reagent to practical peptide synthesis, i.e., choice of a suitable protecting group for arginine and prevention of S-alkylation of methionine. A new arginine derivative, Arg(MBS), introduced by Nishimura and Fujino³⁰⁾ has met the present needs, since MSA removed this protecting group within 60 minutes at 20°, while the nitro⁵⁵⁾ and tosyl⁵⁶⁾ groups employed currently in peptide synthesis resisted the action of this acid. Despite such ease of removal of the MBS group, it was necessary to overcome an unexpected side reaction at tyrosine. The MBS cation, liberated by this acid (as well as by HF), attacked the phenolic group of tyrosine to form Tyr(MBS),⁵⁷⁾ even in the presence of a cation scavenger, anisole; this happenstance was overcome by the use of an alternative scavenger, m- or o-cresol. Recently a more acid labile protecting group for arginine, mesitylene-2-sulphonyl group,⁵⁸⁾ was introduced. However, this new derivative was not ready for use in the present synthesis.

As the 2nd problem, it was found that S-alkylation of methionine in the MSA-anisole system was due to methyl transfer from anisole³¹⁾ and this side reaction could be totally prevented by the use of methionine sulfoxide, Met(O).³⁷⁾ An easy procedure for the preparation of Met(O) by sodium metaperiodate or sodium perborate oxidation provided with sufficient starting material.⁵⁹⁾

An unexpected problem arose with the formation of the sulphoxide of Cys(MBzl) during the peptide synthesis. The chemical nature of the Cys(MBzl) sulphoxide has hitherto not been examined in peptide synthesis. There was an indication that the partial oxidation of Cys(MBzl)⁶⁰⁾ had indeed occurred during the long course of the synthesis and when treated with MSA-anisole, as well as HF, this compound was fully transformed to p-methoxyphenylcysteine,³⁸⁾

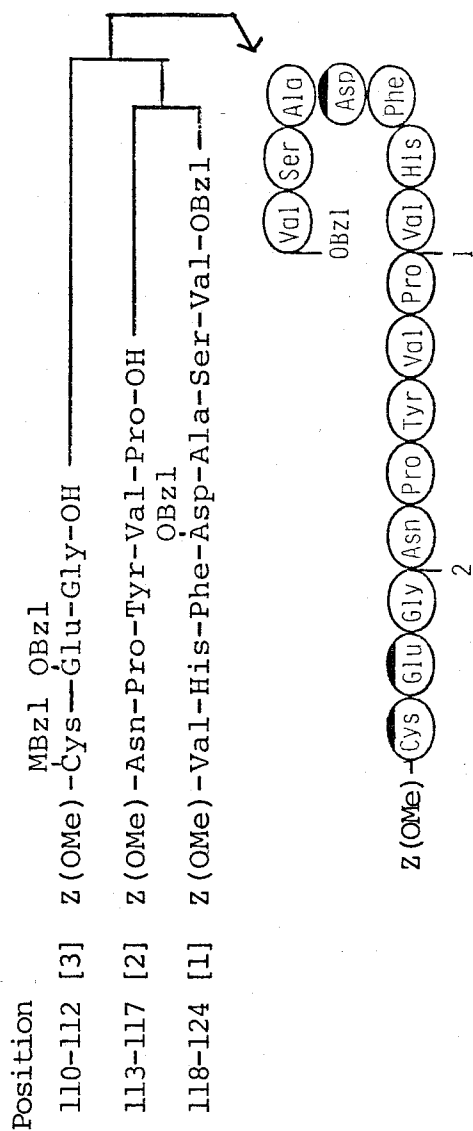
instead of cysteine. Reduction of the sulfoxide of Cys(MBzl) with thiols became necessary prior to the MSA deprotection. This simultaneously reduced Met(O) to methionine. Thus, the protecting group for methionine needed to be removed, prior to removal of the other protecting groups. This new situation prompted to investigate an alternative scavenger, that has no tendency to alkylate methionine. For this and other purposes, m-cresol was selected. The author believes that the use of Met(O) played an important role in preventing the partial S-oxidation during the synthesis and the partial S-alkylation during the N^α-deprotection as mentioned by Iselin.³⁷⁾

With the above strategy and keeping various side reactions in mind, the author undertook the synthesis of RNase A with amino acid derivatives bearing protecting groups removable by MSA, i.e., Asp(OBzl), Glu(OBzl), Lys(Z), Arg(MBS) and Cys(MBzl), in combination with the TFA labile Z(OMe) group⁶¹⁾ as the N^α-protecting group. A reagent, p-methoxybenzyl-8-quinolyl carbonate,⁶²⁾ served to secure sufficient quantities of Z(OMe)-amino acids as starting materials. After performing a preliminary coupling on a small scale, two successive runs were performed in preparative scale at each condensation step to obtain sufficient intermediates. Thus, 4.9 g of the protected S-protein was synthesized. The author herein presents a detailed account of the synthesis of RNase A.

In the first section, the synthesis of the protected pentadecapeptide ester corresponding to the C-terminal portion of RNase (position 110-124) is described. The pentadecapeptide ester, Z(OMe)-Cys(MBzl)-Glu(OBzl)-Gly-Asn-Pro-Tyr-Val-Pro-Val-His-Phe-Asp(OBzl)-Ala-Ser-Val-OBzl, abbreviated as Z(OMe)-(RNase 110-124)-OBzl, was synthesized according to Scheme 1., where three peptide fragments, [1], [2] and [3], served as building blocks. The Merck group^{41-c)} selected also these three units; two hydrazides and the C-terminal heptapeptide with the free carboxyl group, though no experimental details were given.

Scheme 1. Synthetic route to the protected pentadecapeptide ester,

Z(OMe)-(RNase 110-124)-OBzl



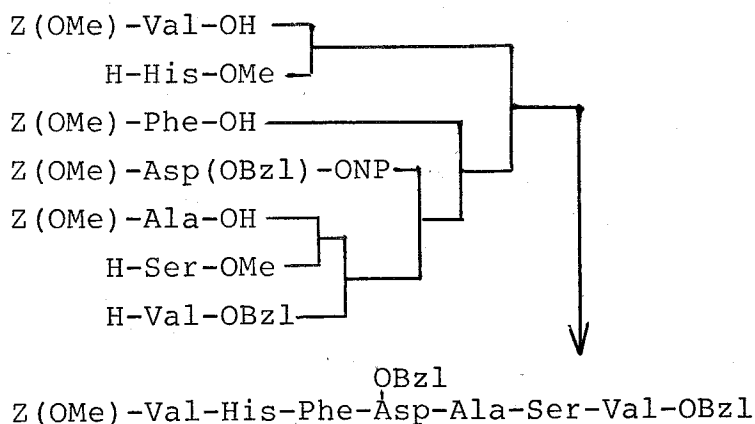
○ Protected amino acid: Asp(OBzl), Glu(OBzl), Cys(MBzl).

| Positions of fragment condensation.

The first block [1], Z(OMe)-Val-His-Phe-Asp(OBzl)-Ala-Ser-Val-OBzl, Z(OMe)-(RNase 118-124)-OBzl, was synthesized according to the scheme 1-a. The C-terminal carboxyl group was, in this synthesis, protected as the benzyl ester. Attempt to prepare Z-Ala-Ser-Val-OH by the azide coupling of Z-Ala-Ser-NHNH₂⁶³⁾ with valine gave poor yield, because of the difficulty involved in bringing valine into an aqueous solution, even in the presence of the theoretical amount of triethylamine. The coupling of Z(OMe)-Ala-Ser-NHNH₂ with H-Val-OBzl was performed to give Z(OMe)-Ala-Ser-Val-OBzl in a satisfactory yield. Prior to each chain elongation, the Z(OMe) group was removed by TFA in the presence of anisole. To the

Scheme 1-a.

Synthetic scheme of the protected heptapeptide ester, Z(OMe)-(RNase 118-124)-OBzl [1].



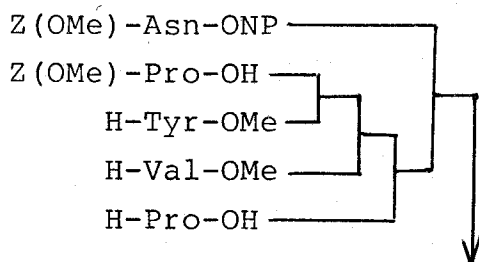
resulting N^α-deprotected peptide, H-Ala-Ser-Val-OBzl in this instance, Z(OMe)-Asp(OBzl)-OH and Z(OMe)-Phe-OH were incorporated in a stepwise manner by the p-nitrophenyl ester procedure⁶⁴⁾ and DCC condensation⁶⁵⁾ respectively and subsequently Z(OMe)-Val-His-NHNH₂ by the Rudinger's azide procedure. The latter hydrazide was prepared by the mixed anhydride procedure⁶⁶⁾ followed by the usual hydrazine treatment of the resulting dipeptide ester, Z(OMe)-Val-

His-OMe. In this step, the DCC condensation is known to give the DCC adduct of histidine,⁶⁷⁾

The fragment [2], Z(OMe)-Asn-Pro-Tyr-Val-Pro-OH, Z(OMe)-(RNase 113-117)-OH, was synthesized according to Scheme 1-b. Construction of the peptide bonds, Val-Pro-Val- (116-118), proved to be considerably difficult, due to the combination of sterically hindered amino acids. Prior to synthesizing this fragment, Val-Pro-Val-His- unit was prepared as a model compound. The DCC condensation between proline and valine is known to proceed smoothly, but not between valine and proline, because of predominant formation

Scheme 1-b

Synthetic scheme of the protected penta-peptide, Z(OMe)-(RNase 113-117)-OH [2].



Z(OMe)-Asn-Pro-Tyr-Val-Pro-OH

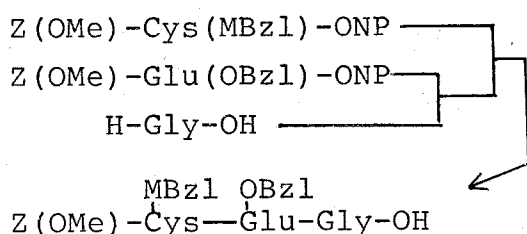
of an acyl urea, a side reaction of DCC.⁶⁸⁾ Thus, it was decided to terminate this fragment at the position of proline (117) and establish the Val-Pro bond by condensation of Z(OMe)-Pro-Tyr-Val-NHNH₂ and proline by the azide procedure. The former tripeptide hydrazide was the one derived from the azide condensation of the known hydrazide, Z(OMe)-Pro-Tyr-NHNH₂,⁶⁹⁾ with H-Val-OMe followed by the usual hydrazine treatment. The resulting tetrapeptide, Z(OMe)-Pro-Tyr-Val-Pro-OH, after the TFA treatment, was submitted to the condensation with Z(OMe)-Asn-OH by the p-nitrophenyl ester method to give the fragment [2], without a sizable amount of the

O-acyl component⁷⁰⁾ at the tyrosine residue.

The fragment [3], Z(OMe)-Cys(MBzl)-Glu(OBzl)-Gly-OH, Z(OMe)-(RNase 110-112)-OH, was synthesized without particular difficulty by the successive p-nitrophenyl ester condensation of Z(OMe)-Glu(OBzl)-OH and Z(OMe)-Cys(MBzl)-OH as shown in Scheme 1-c.

Scheme 1-c.

Synthetic scheme of the protected tripeptide, Z(OMe)-(RNase 110-112)-OH [3].



Two fragments [2] and [3] with the C-terminal proline and glycine respectively were successively condensed with fragment [1] by the pentachlorophenyl trichloroacetate (PCP-O-TCA) procedure⁷¹⁾ without risk of racemization. It was noticed that each reaction was accelerated by the addition of HOBT.⁷²⁾ Each product was purified by the washing and precipitations. Their amino acid ratios in 6N HCl hydrolysates are listed in Table 1, where recovery of phenylalanine was taken as the basis of calculation, since this amino acid occurs once in the C-terminal portion at position 120 and then at positions 46 and 8. Such diagnostic amino acids should be selected from amino acids with minimal composition and located at the C-terminal portion of the molecule. The author believes that such choice of amino acid as the basis of calculation throughout the synthesis is absolutely necessary for obtaining informations about purity of each condensation product, rather than varying amino acid as the basis for convenience. Various synthetic tactics arising in this synthesis of RNase is described according to the

chain elongation.

For structure-function studies on RNase, a number of C-terminal peptides have been synthesized; i.e., a fragment (118-124) in a conventional manner^{73-a)} with different protecting groups from those described herein, the fragments (116-124), (113-124) and (111-124) by the solid method^{73-b)} and analogs of (111-124) also by the solid method.^{73-c)}

Table 1. Amino acid ratios of Z(OMe)-(RNase 110-124)-OBzl and intermediates.

Position Residue	118-124 (7)	113-124 (12)	110-124 (15)
Asp	0.95(1)	2.06(2)	2.03(2)
Ser	0.84(1)	0.87(1)	0.90(1)
Glu			1.07(1)
Pro		2.12(2)	2.19(2)
Gly			0.99(1)
Ala	0.94(1)	0.98(1)	1.02(1)
Val	1.98(2)	2.95(3)	2.98(3)
Tyr		0.97(1)	0.94(1)
Phe	1.00(1)	1.00(1)	1.00(1)
His	0.75(1)	0.78(1)	0.87(1)
Cys			(1)
Recovery	89%	91%	89%

Section 2. Synthesis of the Protected Hexatriaconta-peptide Ester (positions 89-124)

Summary

Commencing with the protected C-terminal pentadecapeptide of bovine pancreatic RNase, Z(OMe)-(RNase 110-124)-OBzl, chain elongation was accomplished to the hexatriacontapeptide, Z(OMe)-(RNase 89-124)-OBzl, by successive azide condensation of six peptide fragments, i.e., Z(OMe)-Val-Ala-NHNH₂ [4], Z(OMe)-Ile-Ile-NHNH₂ [5], Z(OMe)-Asn-Lys(Z)-His-NHNH₂ [6], Z(OMe)-Lys(Z)-Thr-Thr-Gln-Ala-NHNH₂ [7], Z(OMe)-Tyr-Pro-Asn-Cys(MBzl)-Ala-Tyr-NHNH₂ [8], and Z(OMe)-Ser-Ser-Lys(Z)-NHNH₂ [9].

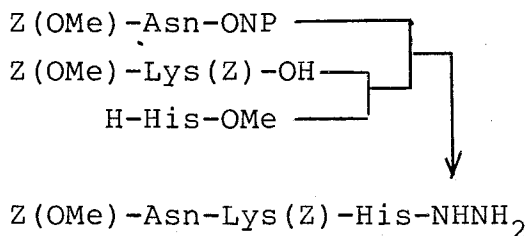
Succeeding to the synthesis of the protected C-terminal pentadecapeptide (position 110-124), its chain elongation to the hexatriacontapeptide (position 89-124) is described in this section. Chain elongation was accomplished by successive azide condensation²¹⁾ of six peptide fragments as shown in Scheme 2.

The positions 106-108 of RNase consists of sterically hindered amino acids, Ile-Ile-Val. Initially, Z(OMe)-Ile-Ile-Val-Ala-OMe was prepared in a stepwise manner by DCC condensation⁶⁵⁾ without difficulty. However, the corresponding hydrazide was a much less soluble compound even in hot DMF or DMSO. Because of this difficulty, it was decided to introduce this tetrapeptide unit in two steps as Z(OMe)-Val-Ala-NHNH₂ [4] and Z(OMe)-Ile-Ile-NHNH₂ [5]. These two hydrazides were obtained by the usual hydrazine treatment of the corresponding esters, prepared by the mixed anhydride procedure⁶⁶⁾ respectively, rather than by the DCC procedure. The latter procedure gave considerable amounts of the acylurea compounds⁶⁸⁾ in both cases.

The fragment, Z(OMe)-Asn-Lys(Z)-His-NHNH₂ [6] (position 103-105), was prepared as shown in Scheme 2-a. Rink and Riniker⁶⁷⁾ pointed

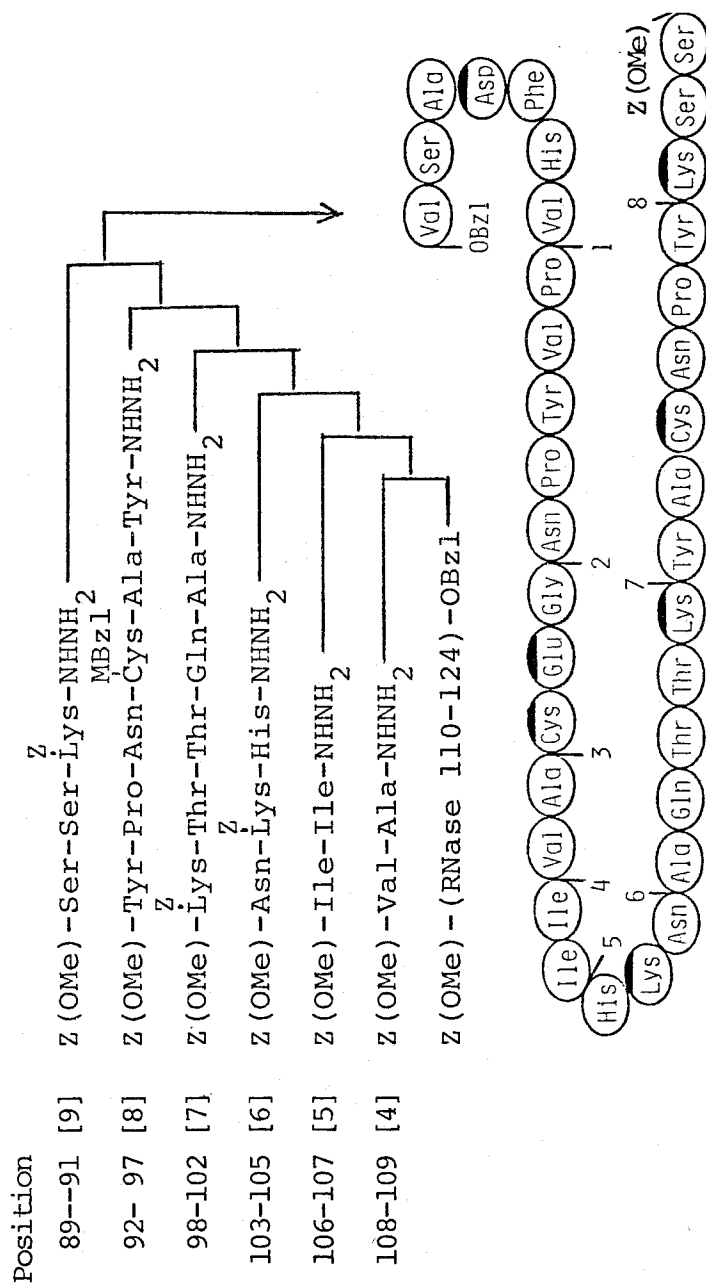
Scheme 2-a.

Synthetic scheme of the protected tripeptide hydrazide, Z(OMe)-(RNase 103-105)-NHNH₂ [6]



out that the DCC condensation of histidine-containing peptide gave a DCC adduct at the imidazole ring. After the DCC condensation of Z(OMe)-Lys(Z)-OH and H-His-OMe, the product was, therefore, treated with methanol and 3% acetic acid to remove this adduct. Z(OMe)-Asn-

Scheme 2. Synthetic route to the protected hexatriacontapeptide ester, Z(OMe)-(RNase 89-124)-OBzl



Protected amino acid: Asp(OBzl), Glu(OBzl), Cys(MBzl), Lys(Z).

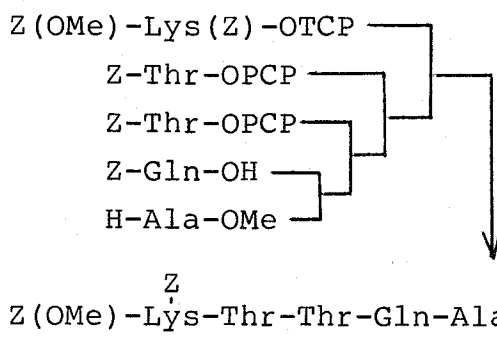
Positions of fragment condensation.

OH was condensed by the NP method⁶⁴⁾ with the TFA treated sample of Z(OMe)-Lys(Z)-His-OMe thus obtained and subsequently the product was converted to [6] by the usual hydrazine treatment.

According to the scheme 2-b, the fragment, Z(OMe)-Lys(Z)-Thr-Thr-Gln-Ala-NHNH₂ [7] (position 98-102), was synthesized starting with Z-Gln-Ala-OMe⁷⁴⁾ prepared by DCC condensation in the presence of HOBT.¹⁹⁾ No CN bond vibration⁷⁵⁾ was observed in the IR spectrum of this starting material. After removing the Z group by catalytic hydrogenolysis, the PCP⁷⁶⁾ and TCP⁷⁷⁾ active ester procedures were employed to introduce threonine and Lys(Z), respectively, since these esters are crystalline compounds.^{78,79)} The resulting pentapeptide ester, Z(OMe)-Lys(Z)-Thr-Thr-Gln-Ala-OMe, was smoothly converted to [7] by hydrazine in the usual manner.

Scheme 2-b.

Synthetic scheme of the protected pentapeptide hydrazide, Z(OMe)-(RNase 98-102)-NHNH₂ [7].

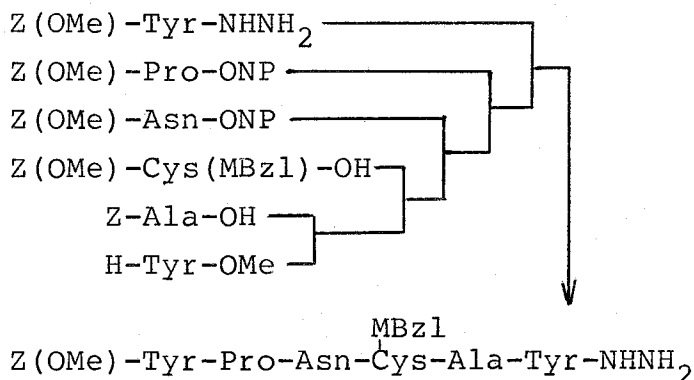


Next, the author tried to elongate the peptide chain by adding two subunits, Z(OMe)-Pro-Asn-Cys(MBzl)-Ala-Tyr-NHNH₂ and Z(OMe)-Ser-Ser-Lys(Z)-Tyr-NHNH₂. However, the preliminary findings indicated that the latter azide, even in five-fold excess, did not bring the condensation in completion with the sterically hindered Pro-terminal peptide, a relatively large amino component of sequence 93-124. Thus, the next two fragments were alternatively syn-

thesized; i.e., Z(OMe)-Tyr-Pro-Asn-Cys(MBzl)-Ala-Tyr-NHNH₂ [8] and Z(OMe)-Ser-Ser-Lys(Z)-NHNH₂ [9]. The former hydrazide was synthesized in a stepwise manner starting with Z-Ala-Tyr-OMe⁸⁰⁾ as shown in Scheme 2-c. Z(OMe)-Cys(MBzl)-OH was introduced by DCC, subsequently two amino acids, Z(OMe)-Asn-OH and Z(OMe)-Pro-OH, by the NP method and finally Z(OMe)-Tyr-NHNH₂ by the azide method. The resulting hexapeptide ester was converted to [8] in the usual manner.

Scheme 2-c.

Synthetic scheme of the protected hexapeptide hydrazide, Z(OMe)-(RNase 92-97)-NHNH₂ [8].

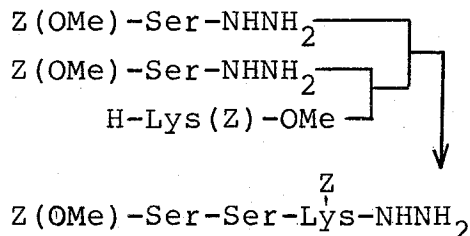


Fragment [9] (position 89-91) was prepared by two successive azide condensation of Z(OMe)-Ser-NHNH₂ with H-Lys(Z)-OMe followed by treatment of the resulting tripeptide ester, Z(OMe)-Ser-Ser-Lys(Z)-OMe, with hydrazine (Scheme 2-d). Previously, Z(OMe)-Ser-OPCP was used for the preparation of the aforementioned tetrapeptide hydrazide. However some difficulty was encountered in the preparation of the starting material, Z(OMe)-Ser-OH,⁶¹⁾ due to its high solubility in water.

Six peptide hydrazides, the purities of which were assessed by elemental analysis and amino acid analysis after acid hydrolysis, were then assembled successively by Rudinger's azide procedure. The possibility of a certain degree of racemization occurring during

Scheme 2-d.

Synthetic scheme of the protected tripeptide hydrazide, Z(OMe)-(RNase 89-91)-NHNH₂ [9].



the azide coupling was pointed out by several authors.⁸¹⁾ However, on the basis of available informations, it is still considered that the azide method is the method of choice for obtaining peptides with high optical purity. Particularly, the peptides were synthesized without masking the hydroxyl group of serine and threonine and the phenolic group of tyrosine. Bodanszky et al.⁷⁰⁾ reported the over activation of the active ester procedure, which took place at these functional groups in the presence of histidine. It was felt safer to carry out the further synthesis by persuing the azide procedure, as demonstrated by Denkwalter et al.,⁴¹⁾ since much less risk seems to be involved in this procedure, which can be performed with cooling.

Among condensation reactions involved in this section, the point with which concerned was the bond formation between isoleucine and valine (position 107-108) via the azide condensation of Z(OMe)-Ile-Ile-NHNH₂ [5] with the Val-terminal amino component (position 108-124), because of steric hindrance of these amino acids. Indeed, conversion of Z(OMe)-Ile-Ile-OMe to the corresponding hydrazide needed a slight warming at 50°, but its azide reaction went smoothly in better than expected yield (79%). Acid hydrolysis of the protected peptide, abbreviated as Z(OMe)-(RNase 106-124)-OBzl, gave a low recovery of isoleucine, as predicted from earlier observations.^{41-c)} A similar phenomenon⁸²⁾ was also noted dur-

ing the synthesis of basic trypsin inhibitor, which has the identical Ile-Ile-sequence.⁸³⁾ Thus, 72 hr's hydrolysis was performed at this stage to ascertain the satisfactory incorporation of this dipeptide unit. On analysis of this product, the presence of D-allo-Ile was detected (2.3%). Acid hydrolysis of the above starting hydrazide did not give any detectable amount of the allo-compound, which is known as the guide substance of racemization test.⁸⁴⁾ It had to be admitted that racemization could occur, not only in the above coupling step, but also in every azide condensation of peptide fragments usually in less than 0.04%.^{81-c)}

Every condensation was performed with ca. 1.3-2.5 fold excess of an azide component, until the solution became negative to ninhydrin. The progress of the reaction was examined by Shimadzu dual wavelength tlc scanner. As a solvent, a mixture of DMF-DMSO or DMF-HMPA was employed, depending on the solubility of amino components. Especially, after incorporation of Val-Ala unit [4], succeeding condensations of the fragments, from [5] to [9], were performed in such a solvent system. Even under these conditions, certain improvement of solubility was noted, after incorporation of fragments bearing protecting groups, such as Lys(Z) and Cys(MBzl). Excess peptide azide could be removed by repeated precipitation from DMF or a mixture of DMF and DMSO with methanol or ethyl acetate. Thus, coupling yield lay within 72 to 93%. The purity of protected intermediates were assessed by tlc and amino acid analysis (Table 2). The recovery of phenylalanine was taken as the basis of calculation, as mentioned in the preceding section. Elemental analysis seems to offer little information about the purity of relatively large peptides; however, this analysis was performed to obtain as much information as possible about the homogeneity.

The docosapeptide synthesized (position 103-124) by Jenkins et al.^{41-c)} was similar to the one described here with the exception of the protecting groups employed.

Table 2. Amino acid ratios of Z(OMe)-(RNase 89-124)-OBzl and intermediates.

Position Residue	108-124 (17)	106-124 (19)*	103-124 (22)	98-124 (27)	92-124 (33)	89-124 (36)
Asp	1.97(2)	2.02(2)	3.09(3)	3.09(3)	4.24(4)	4.20(4)
Thr				2.04(2)	1.80(2)	1.60(2)
Ser	0.89(1)	0.77(1)	0.78(1)	0.82(1)	0.72(1)	2.47(3)
Glu	1.06(1)	1.08(1)	1.12(1)	2.34(2)	2.26(2)	2.08(2)
Pro	2.00(2)	2.18(2)	2.17(2)	2.20(2)	3.05(3)	2.62(3)
Gly	1.05(1)	1.01(1)	1.09(1)	1.06(1)	1.08(1)	1.08(1)
Ala	2.03(2)	2.01(2)	1.91(2)	2.98(3)	4.18(4)	3.89(4)
Val	4.09(4)	3.89(4)*	3.76(4)	3.85(4)	3.86(4)	4.03(4)
Ile		1.84(2)	1.82(2)	1.76(2)	1.86(2)	1.84(2)
Tyr	0.94(1)	0.97(1)	0.91(1)	0.99(1)	2.97(3)	2.65(3)
Phe	1.00(1)	1.00(1)	1.00(1)	1.00(1)	1.00(1)	1.00(1)
Lys			1.13(1)	2.10(2)	1.81(2)	2.99(3)
His	0.83(1)	0.88(1)	1.84(2)	1.98(2)	1.78(2)	1.82(2)
Cys	(1)	(1)	(1)	(1)	(2)	(2)
Recovery	92%	97%	94%	84%	87%	86%

* Ile 2.02 in 72 hr's hydrolysate.

Section 3. Synthesis of the Protected Hexapentacontapeptide Ester (positions 69-124)

Summary

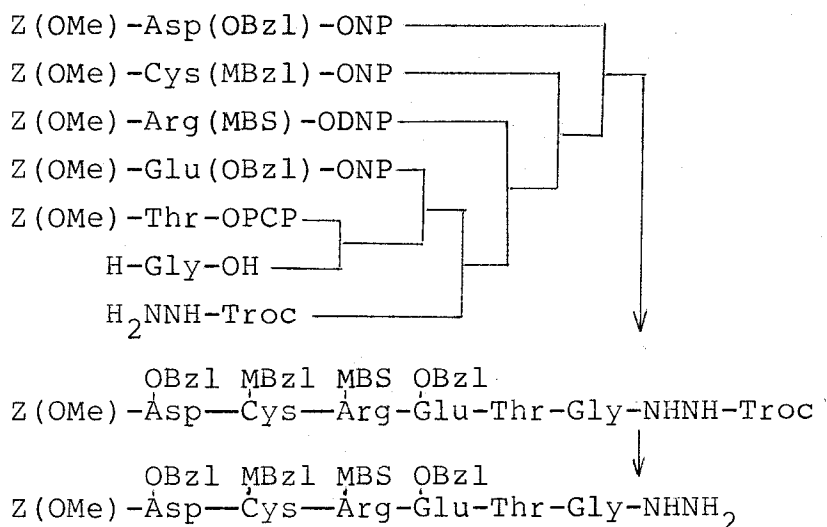
Commencing with the protected hexatriacontapeptide, corresponding to the sequence of bovine pancreatic RNase A, Z(OMe)-(RNase 89-124)-OBzl, chain elongation was carried out to the hexapentacontapeptide stage, Z(OMe)-(RNase 69-124)-OBzl, by successive azide condensation of six peptide fragments; i.e., Z(OMe)-Asp(OBzl)-Cys(MBzl)-Arg(MBS)-Glu(OBzl)-Thr-Gly-NHNH-Troc [10], Z(OMe)-Ser-Ile-Thr-NHNH₂ [11], Z(OMe)-Ser-Thr-Met(O)-NHNH₂ [12], Z(OMe)-Gln-Ser-Tyr-NHNH₂ [13], Z(OMe)-Asn-Cys(MBzl)-Tyr-NHNH₂ [14] and Z(OMe)-Gln-Thr-NHNH₂ [15]. The Troc group was removed from the fragment [10] by treatment with Zn, prior to condensation. With N-methylpyrrolidone-5% H₂O as eluent, gel-filtration on Sephacryl S-200 was employed for purification of Z(OMe)-(RNase 69-124)-OBzl contaminated with the acyl component used in excess during the coupling.

Following the synthesis of the protected hexatriacontapeptide ester corresponding to bovine pancreatic RNase A (position 89-124), the author herein describes further chain elongation to the protected hexapentacontapeptide ester, Z(OMe)-(RNase 69-124)-OBzl, performed by successive condensation of six peptide fragments, from [10] to [15], as shown in Scheme 3.

The protected hexapeptide hydrazide derivative, Z(OMe)-Asp(OBzl)-Cys(MBzl)-Arg(MBS)-Glu(OBzl)-Thr-Gly-NHNH-Troc [10], (position 83-88), was synthesized according to Scheme 3-a. The glycine residue at position 88 can be chosen as the racemization-free condensation point. Therefore, it was initially planned to prepare the protected hexapeptide without protection of the carboxyl group and couple this unit by DCC in the presence of HOBT¹⁹⁾ to the relatively large amino component, H-(89-124)-OBzl. However, considering

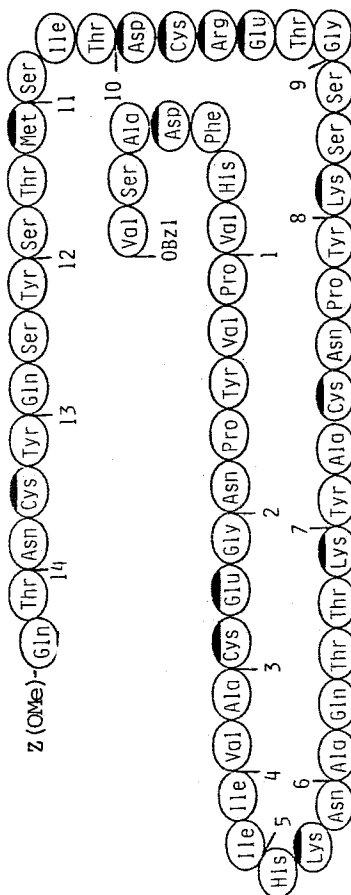
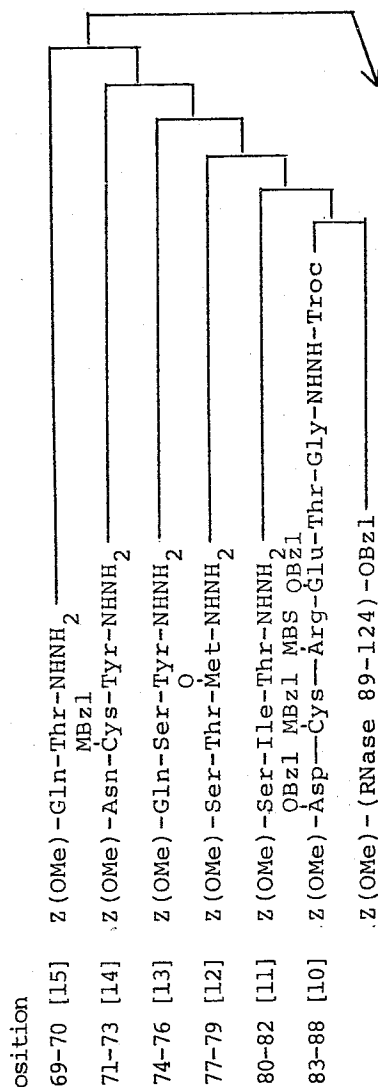
Scheme 3-a.

Synthetic scheme of the protected hexapeptide hydrazide derivative, Z(OMe)-(RNase 83-88)-NHNH-Troc [10].



Scheme 3. Synthetic route to the protected hexapentacontapeptide ester,

Z(OMe)-(RNase 69-124)-OBzl



Protected amino acids: Glu(OBzl), Cys(MBzl), Arg(MBS), Lys(Z), Asp(OBzl), Met(O).

| Positions of fragment condensation.

the various preferable features of the Rudinger's azide coupling procedure²¹⁾ in the present synthesis, it was decided to adopt a substituted hydrazine to prepare this hydrazide containing Asp(OBzl) and Glu(OBzl), since the usual hydrazinolysis of esters can not be applied. Troc-NHNH₂ introduced by Kiso and Yajima in 1971⁸⁵⁾ met the present requirements, since the Troc group can be removed by treatment with Zn,⁸⁶⁾ without affecting the side chain protecting groups; Z(OMe), Z, Bzl, MBzl, and MBS.³⁰⁾ The known substituted hydrazines, Z-NHNH₂,⁸⁷⁾ Boc-NHNH₂,⁸⁸⁾ Tri-NHNH₂,⁸⁹⁾ Picoc-NHNH₂⁹⁰⁾ did not fulfil this requirement.

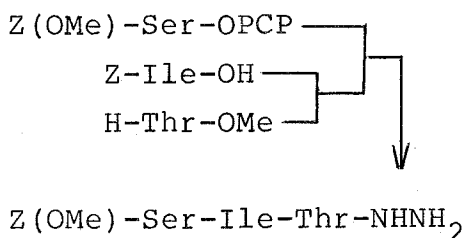
Thus, Z(OMe)-Glu(OBzl)-Thr-Gly-OH prepared in a stepwise manner was converted quantitatively, by the DCC plus HOBT condensation¹⁹⁾ with Troc-NHNH₂, to the crystalline hydrazide derivative, Z(OMe)-Glu(OBzl)-Thr-Gly-NHNH-Troc. This fragment, after N^α-deprotection with TFA,⁶¹⁾ was allowed to condense with Z(OMe)-Arg(MBS)-OH,³⁰⁾ a new agrinine derivative bearing the protecting group removable by MSA,²²⁾ by means of the DNP method.⁹¹⁾ By monitoring on tlc, the reaction was performed in 81% yield without isolation of the active ester. This is the first time during the synthesis, that an arginine residue has been incorporated into the chain. To the resulting protected tetrapeptide, Z(OMe)-Arg(MBS)-Glu(OBzl)-Thr-Gly-NHNH-Troc, two amino acid derivatives, Z(OMe)-Cys(MBzl)-OH and Z(OMe)-Asp(OBzl)-OH, were successively introduced by the NP active ester procedure⁶⁴⁾ to give the protected hexapeptide hydrazide derivative [10], from which the Troc group was smoothly removed by Zn in acetic acid. The last trace of contaminated zinc acetate was removed by EDTA. The purity of Z(OMe)-Asp(OBzl)-Cys(MBzl)-Arg(MBS)-Glu(OBzl)-Thr-Gly-NHNH₂ was confirmed by tlc, elemental and amino acid analyses.

The fragment, Z(OMe)-Ser-Ile-Thr-NHNH₂ [11] (position 80-82), was prepared using the known dipeptide, Z-Ile-Thr-OMe,⁹²⁾ and an available active ester, Z(OMe)-Ser-OPCP, without particular diffi-

culty as shown in Scheme 3-b.

Scheme 3-b.

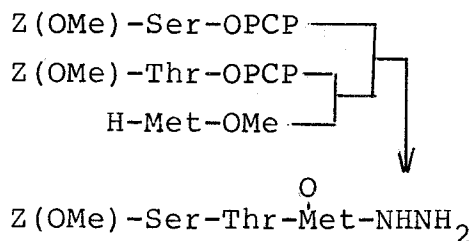
Synthetic scheme of the protected tripeptide hydrazide, Z(OMe)-(RNase 80-82)-NHNH₂ [11].



In the next fragment, Z(OMe)-Ser-Thr-Met(O)-NHNH₂ [12] (position 77-79), the methionine residue was protected as the sulfoxide³⁷⁾ according to the previously mentioned strategy. In addition to the two hydrophilic amino acid residues, serine and threonine, Met(O) derivatives are usually very water soluble compounds. Therefore, it was decided to oxidize methionine at the last stage of the synthesis, instead of starting with Z(OMe)-Met(O)-OMe. The tripeptide ester, Z(OMe)-Ser-Thr-Met-OMe prepared using available active esters, Z(OMe)-Thr-OPCP and Z(OMe)-Ser-OPCP, was exposed to the action of tetrachloroauric (II) acid according to Bordignon et al.⁹³⁾ as shown in Scheme 3-C. Oxidation with hydrogen peroxide³⁷⁾ is known to yield Met(O) with a slight contamination of sulphone; a superior oxidant, sodium perborate,⁵⁹⁾ was not available at this time. The author was interested in this reagent, which stereospecifically oxidizes the sulphur atom of methionine. When examined on tlc, the reaction seemed to be completed within 3 hr at 40°. However, because of its water solubility, it was not possible to isolate the desired compound in an analytically pure form. The crude product contaminated with some inorganic salt was converted to the corresponding hydrazide [12], which permitted to ascertain its homogeneity.

Scheme 3-c.

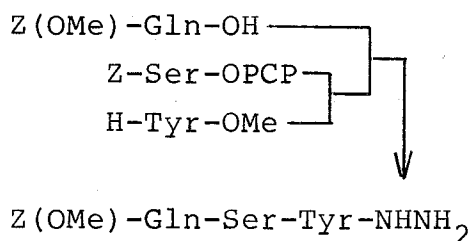
Synthetic scheme of the protected tripeptide hydrazide, Z(OMe)-(RNase 77-79)-NHNH₂ [12].



The next fragment, Z(OMe)-Gln-Ser-Tyr-NHNH₂ [13] (position 74-76), was prepared starting with the known dipeptide ester, Z-Ser-Tyr-OMe,³⁵⁾ which, after hydrogenolysis, was condensed, in an ice-bath, with Z(OMe)-Gln-OH by DCC in the presence of HOBT. The purified tripeptide, Z(OMe)-Gln-Ser-Tyr-OMe, free from a dehydro derivative,⁷⁵⁾ was converted to [13] in the usual manner as shown in Scheme 3-d.

Scheme 3-d.

Synthetic scheme of the protected tripeptide hydrazide, Z(OMe)-(RNase 74-76)-NHNH₂ [13].

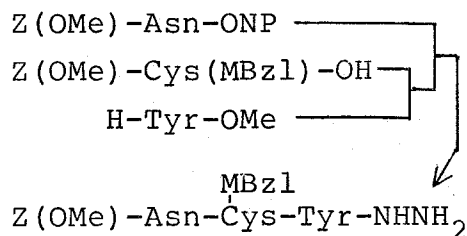


Next, it was attempted to introduce the sequence 69-73 as one unit by condensation of Z(OMe)-Gln-Thr-NHNH₂ [15] (position 69-70) with H-Asn-Cys(MBzl)-Tyr-OMe. The former dipeptide hydrazide [15] was found to be a compound less soluble in DMF. Thus, it was decided to use building blocks in a stepwise manner. Z(OMe)-Asn-Cys(MBzl)-Tyr-NHNH₂ [14] (position 71-73) was prepared according

to the scheme 3-e. Z(OMe)-Cys(MBzl)-Tyr-OMe was prepared by the DCC condensation procedure as usual and this, after the TFA treatment, was allowed to react with Z(OMe)-Asn-ONP. The resulting tripeptide ester was converted to [14] as usual.

Scheme 3-e.

Synthetic scheme of the protected tripeptide hydrazide, Z(OMe)-(RNase 71-73)-NHNH₂ [14].



In order to synthesize Z(OMe)-(RNase 69-124)-OBzl, six fragments thus obtained were assembled according to Scheme 3. Each azide condensation was performed with 2.5 to 3.5 fold excess of the azide component in a solvent system in most instances of DMSO-DMF. Compared with the condensations described in the preceding section, the amount of the azide was thus increased to bring every condensation to completion. After repeated precipitation from DMSO with methanol, the desired products were isolated in thin layer chromatographically pure form and their homogeneities were assessed at each step by amino acid analysis, in which the recovery of phenylalanine was used as a standard (Table 3).

In the course of successive azide condensations, there existed, however, some problems of removing the excess unreacted azide component. It is worthwhile to mention that estimation of purity by amino acid analysis is often difficult, as were found in the case of fragments [11] and [12]. Serine and threonine decomposed to some extent during the acid hydrolysis and Met(O) was mostly converted to methionine. Isoleucine (3 moles) gave a low recovery, because of

Table 3. Amino acid ratios of Z(OMe)-(RNase 69-124)-OBzl and intermediates.

Position Residue	83-124 (42)	80-124 (45)	77-124 (48)	74-124 (51)	71-124 (54)	69-124 (56)
Asp	5.21(5)	5.18(5)	5.14(5)	5.09(5)	6.11(6)	6.14(6)
Thr	2.76(3)	3.90(4)	4.87(5)	4.86(5)	4.83(5)	5.76(6)
Ser	2.47(3)	3.01(4)	3.92(5)	4.50(6)	4.48(6)	4.51(6)
Glu	3.12(3)	3.01(3)	3.06(3)	4.10(4)	4.09(4)	5.13(5)
Pro	2.90(3)	2.79(3)	2.81(3)	2.77(3)	2.79(3)	2.76(3)
Gly	2.22(2)	2.14(2)	2.16(2)	2.11(2)	2.13(2)	2.17(2)
Ala	4.14(4)	3.99(4)	3.94(4)	3.91(4)	3.96(4)	3.98(4)
Val**	3.94(4)	3.88(4)	3.88(4)	3.89(4)	3.90(4)	3.91(4)
Met			0.67(1)	0.64(1)	0.69(1)	0.67(1)
Ile	2.08(2)	2.41(3)	2.39(3)	2.47(3)	2.42(3)	2.44(3)
Tyr	2.72(3)	3.21(3)	3.25(3)	4.34(4)	5.31(5)	5.33(5)
Phe	1.00(1)	1.00(1)	1.00(1)	1.00(1)	1.00(1)	1.00(1)
Lys	2.93(3)	3.18(3)	3.20(3)	3.19(3)	3.22(3)	3.24(3)
His	2.12(2)	1.71(2)	1.69(2)	1.73(2)	1.72(2)	1.74(2)
Arg	1.01(1)	0.90(1)	0.92(1)	0.91(1)	0.90(1)	0.89(1)
Cys	(3)	(3)	(3)	(3)	(4)	(4)
	89%	91%	87%	87%	89%	88%

* 72 hr's hydrolysis.

** Met(O) was not calculated.

the presence of Ile-Ile bond (position 106-107), which resisted even 72 hr's hydrolysis.^{41-c, 82)} Thus, after these two coupling steps, ratios of these newly incorporated amino acids to phenylalanine had some uncertainty. When a preliminary run was carried out, it was noticed that after two successive condensations of [13] and [14], recoveries of aspartic acid, tyrosine and glutamic acid were higher than those predicted by theory and that of threonine as well, after condensation of [15]. Each product exhibited some tailing on tlc. Simple precipitation procedure seemed hopeless for yielding a pure compound. Gel-filtration on Sephadex LH-20 or 60 had proven to be a useful tool for purification of protected peptides on previous occasions. But, as the molecular weight of the peptides increased as in the case of Z(OMe)-(RNase 69-124)-OBzl, LH-20 or 60 proved to be ineffective. Galpin et al.⁹⁴⁾ recommended the use of Enzacryl K-2 (DMF or NMP) and Sephadex G-50 (HMPA-5% H₂O) for larger peptides. In the present studies, it was found that partial separation of a contaminant could be achieved by the use of Sepharose CL-6B (DMSO-5% H₂O) or even better by Sephacryl S-200 (NMP-5% H₂O) (Fig. 2). Acid hydrolysis of the main product obtained in the latter gave amino acid ratios predicted by theory; the material obtained from the side peak revealed the presence of amino acids consisting predominantly of positions 69-82 (Table 3').

The result indicated that such contamination was initiated by successive chain elongation of Ser-Ile-Thr [11], which was failed to remove completely during the purification of Z(OMe)-(RNase 80-124)-OBzl. It showed that even a small azide can give occasionally a rearrangement product which is difficult to remove by single precipitation procedure.

Referring to such observation in a preliminary run, next preparative runs were performed with particular care for purification of Z(OMe)-(RNase 80-124)-OBzl, as well as Z(OMe)-(RNase 77-124)-OBzl, taking the isoleucine recovery of the purified sample of

Fig. 2.

Gel-filtration of the crude sample of Z(OMe)-(RNase 69-124)-OBzl
on Sepharose CL-6B

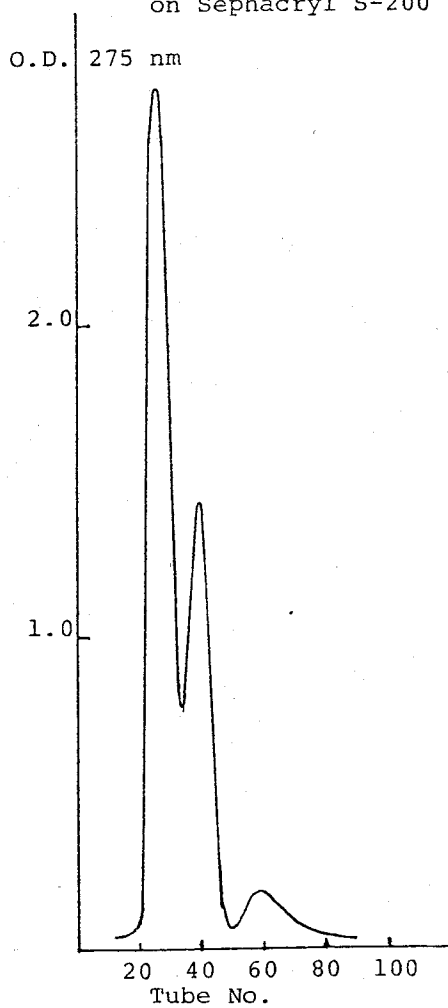
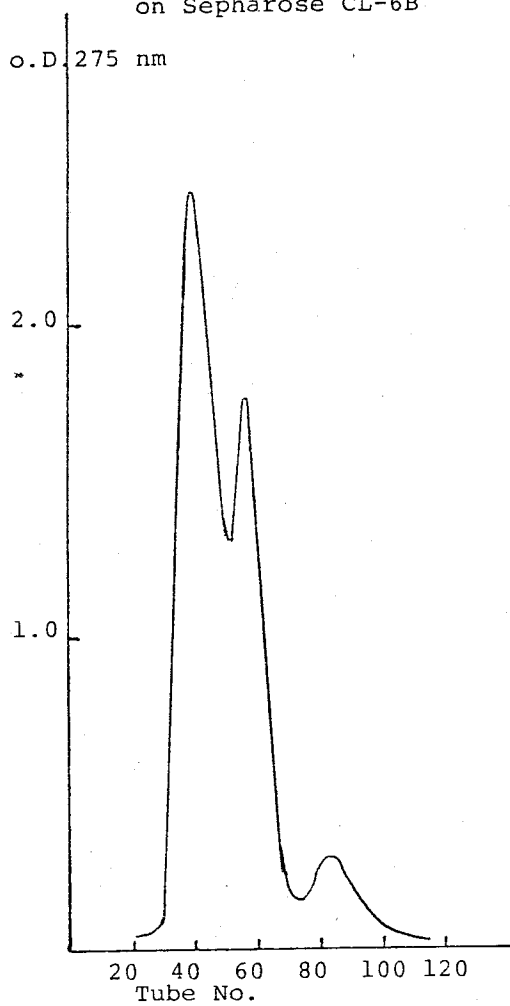


Table 3'. Amino acid analysis of Z(OMe)-(RNase 69-124)-OBzl purified by gel-filtration and crude intermediates.

Position Residue	80-124 (45)	77-124 (48)	74-124 (51)	71-124 (54)	69-124 (56)	Sephacryl S-200 Peak 1	Sephacryl S-200 Peak 2
Asp	5.20(5)	5.10(5)	4.89(5)	6.24(6)	6.47(6)	6.18	11.52
Thr	3.55(4)	5.02(5)	5.00(5)	5.00(5)	6.42(6)	5.62	18.31
Ser	3.12(4)	4.69(5)	5.76(6)	5.27(6)	5.36(6)	4.42	10.76
Glu	3.31(3)	3.26(3)	4.96(4)	4.77(4)	6.09(5)	5.06	17.35
Pro	2.96(3)	2.98(3)	2.80(3)	2.86(3)	2.69(3)	2.69	2.37
Gly	2.32(2)	2.22(2)	2.17(2)	2.16(2)	2.11(2)	2.13	1.78
Ala	4.26(4)	4.07(4)	3.97(4)	3.90(4)	3.76(4)	4.05	3.65
Val	4.02(4)	3.69(4)	3.49(4)	3.47(4)	3.57(4)	3.91	3.49
Met		1.08(1)	0.94(1)	0.94(1)	0.92(1)	0.72	4.79
Ile	2.85(3)	2.68(3)	2.62(3)	2.68(3)	2.53(3)	2.51	2.52
Tyr	2.90(3)	2.73(3)	4.04(4)	5.17(5)	5.41(5)	5.23	17.09
Phe	1.00(1)	1.00(1)	1.00(1)	1.00(1)	1.00(1)	1.00	1.00
Lys	2.86(3)	3.21(3)	2.75(3)	2.85(3)	2.82(3)	2.99	2.76
His	1.85(2)	1.89(2)	1.62(2)	1.70(2)	1.68(2)	1.72	1.49
Arg	1.03(1)	1.06(1)	1.07(1)	1.07(1)	0.97(1)	0.97	1.07
Cys	(3)	(3)	(3)	(4)	(4)		
Recovery	83%	85%	89%	88%	88%	89%	89%

Z(OMe)-(RNase 69-124)-OBzl (Ile/Phe =2.4, after 48 hr's hydrolysis) as a marker. Though a sharp single spot on tlc in the solvent system of n-BuOH-AcOH-AcOEt-H₂O (1:1:1:1, v/v) is still one of the guide for examining routinely the purity of protected intermediates, the author decided to examine the purity, in addition to amino acid analysis, by column chromatography on Sephacryl S-200 at the stage of Z(OMe)-(RNase 69-124)-OBzl. Here is the good check point for establishment of purity of synthetic peptides being carried on by successive condensation from the C-terminal end. A promising tool for purification of relatively large protected peptides became now available, when discrepancy between observed and theoretical values in amino acid compositions is noted. Thus, with very much confidence, the author was able to prepare 19 g of Z(OMe)-(RNase 69-124)-OBzl with a high degree of homogeneity.

Section 4. Synthesis of the Protected Tetraoctacontapeptide Ester (Positions 41-124)

Summary

Commencing with the protected hexapentacontapeptide corresponding to the sequence of bovine pancreatic RNase A, Z(OMe)-(69-124)-OBzl, chain elongation was carried out to the tetraoctacontapeptide, Z(OMe)-(RNase 41-124)-OBzl, by successive azide condensations of seven peptide fragments; i.e., Z(OMe)-Cys(MBzl)-Lys(Z)-Asn-Gly-NHNH-Troc [16], Z(OMe)-Asn-Val-Ala-NHNH₂ [17], Z(OMe)-Val-Cys(MBzl)-Ser-Gln-Lys(Z)-NHNH₂ [18], Z(OMe)-Asp(OBzl)-Val-Gln-Ala-NHNH-Troc [19], Z(OMe)-Glu(OBzl)-Ser-Leu-Ala-NHNH-Troc [20], Z(OMe)-Phe-Val-His-NHNH₂ [21], and Z(OMe)-Lys(Z)-Pro-Val-Asn-Thr-NHNH₂ [22]. The Troc group was removed from the fragments, [16], [19] and [20], by treatment with Zn, prior to each condensation. TLC assessment of the homogeneity of the products was not possible, due to lack of a suitable solvent systems. Ratios of newly incorporated amino acids in the acid hydrolysate to that of phenylalanine, however, gave an important clue to purity. The purity of Z(OMe)-(RNase 41-124)-OBzl was confirmed by gel-filtration on Sephacryl S-200.

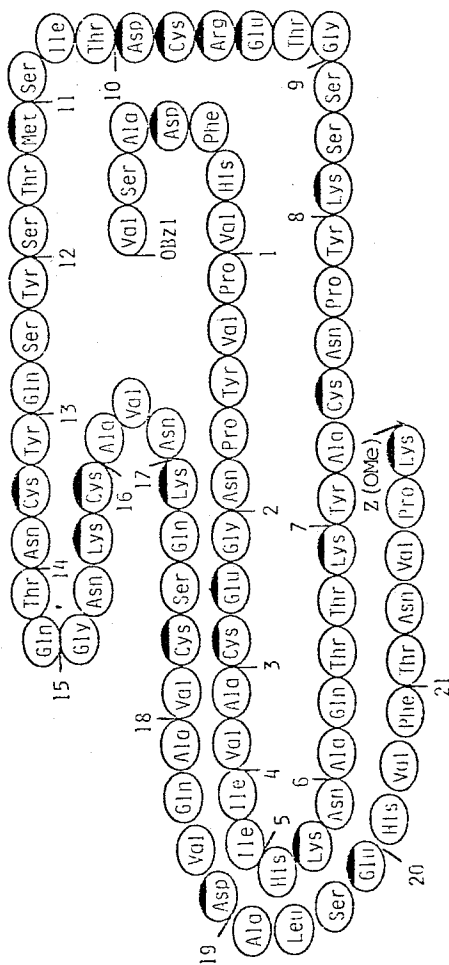
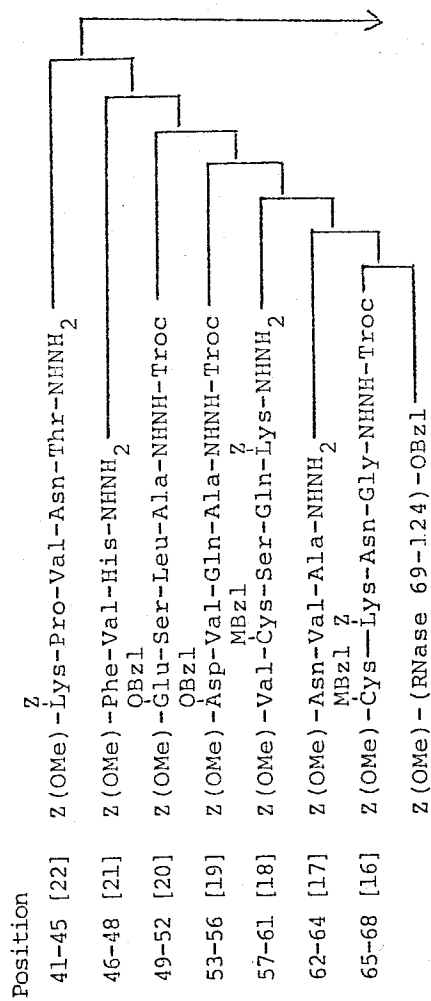
In the preceding section, the synthesis of the protected hexapentacontapeptide ester, Z(OMe)-(RNase 69-124)-OBzl, was described together with column chromatographic purification procedure for the protected peptides on Sephacryl S-200. In this section, the author describes the synthesis of the protected tetraoctacontapeptide ester, Z(OMe)-(RNase 41-124)-OBzl, which was obtained by further chain elongation with seven peptide fragments as shown in Scheme 4.

The selected tetrapeptide fragment, Z(OMe)-Cys(MBzl)-Lys(Z)-Asn-Gly-NHNH-Troc [16] (position 65-68), contains the Asn-Gly bond. As pointed out by Gráf et al.⁹⁵⁾ and Riniker et al.⁹⁶⁾, this particular Asn residue has a great tendency to undergo preferential deamination under alkaline conditions. This fact led to the structural revisions of porcine and human adrenocorticotropins.^{95,96)} This indicates that the hydrazide can not be obtained unambiguously by the usual hydrazine treatment of the corresponding ester. Thus the substituted hydrazine, Troc-NHNH₂⁸⁵⁾ mentioned previously, was applied to the synthesis of this fragment. As shown in Scheme 4-a, Z(OMe)-Asn-ONP was allowed to condense with H-Gly-NHNH-Troc.⁹⁷⁾ The resulting dipeptide, Z(OMe)-Asn-Gly-NHNH-Troc, was treated with TFA⁶¹⁾ and, after neutralization with Et₃N, was coupled to Z(OMe)-Lys(Z)-OH via the 5-chloro-8-quinolyl ester procedure⁹⁸⁾ to give Z(OMe)-Lys(Z)-Asn-Gly-NHNH-Troc. This compound was alternatively prepared by the DCC-HOBT condensation¹⁹⁾ of Troc-NHNH₂ with Z(OMe)-Lys(Z)-Asn-Gly-OH prepared similarly, but starting with glycine. In the latter procedure, the yield of Z(OMe)-Asn-Gly-OH was only 38%. Condensation of Z(OMe)-Cys(MBzl)-OH with the TFA treated sample of the above tripeptide derivative by the NP method⁶⁵⁾ afforded [16], from which the Troc group was removed by Zn to yield the protected tetrapeptide hydrazide, Z(OMe)-Cys(MBzl)-Lys(Z)-Asn-Gly-NHNH₂.

The same tetrapeptide hydrazide unit was selected by Jenkins et al.,^{41-c)} for the preparation of Boc-(RNase 65-85)-OMe. Although they mentioned the base-lability of the Asn-Gly bond, hydrazine

Scheme 4. Synthetic route to the protected tetraoctapeptide ester,

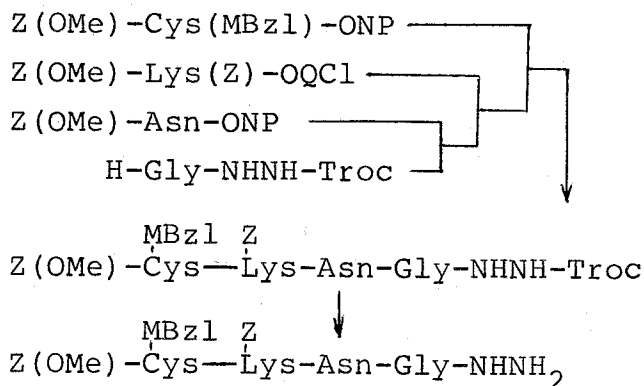
Z(OMe)-(RNase 41-124)-OBzl



Protected amino acid: Asp(OBzl), Glu(OBzl), Lys(Z), Arg(MBS), Met(O).

| Positions of fragment condensation.

Synthetic scheme of the protected tetrapeptide hydrazide derivative, Z(OMe)-(RNase 65-68)-NHNH-Troc [16]



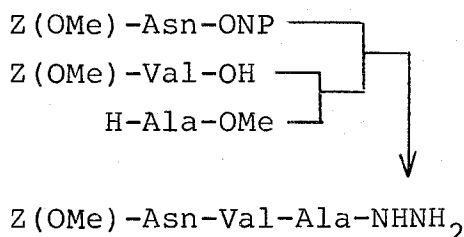
treatment of the tetrapeptide ester was performed and again the above heneicosapeptide ester was exposed to hydrazine to prepare the corresponding hydrazide. Independently, alternative syntheses of the fragments, 65-68 and 65-72, were reported.⁹⁹⁾ After the synthesis of fragment [16], the author noted a report of Meienhofer et al.,¹⁰⁰⁾ in which protected peptides with the free carboxyl group were converted to the hydrazides by DCC and hydrazine in the presence of HOBT.¹⁹⁾ The author is convinced that the procedure employed above is the most suitable method for the preparation of base-sensitive hydrazides, because the risk of deamination accompanying exposure of such peptides to strongly basic hydrazine could be eliminated.

The subsequent fragment, Z(OMe)-Asn-Val-Ala-NHNH₂ [17] (position 62-64), was prepared by condensation of Z(OMe)-Asn-ONP with H-Val-Ala-OMe derived from the Z(OMe)-derivative described in Section 2, followed by the usual hydrazine treatment without particular difficulty as shown in Scheme 4-b.

In order to synthesize the protected pentapeptide hydrazide, Z(OMe)-Val-Cys(MBzl)-Ser-Gln-Lys(Z)-NHNH₂ [18] (position 57-61),

Scheme 4-b.

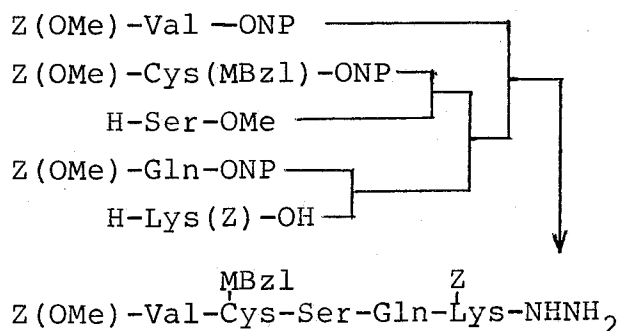
Synthetic scheme of the protected tripeptide hydrazide, Z(OMe)-(RNase 62-64)-NHNH₂ [17]



two dipeptide units, Z(OMe)-Cys(MBzl)-Ser-NHNH₂ and H-Gln-Lys(Z)-OH prepared by the NP method respectively, were joined together by the Rudinger's azide procedure, as shown in Scheme 4-c. Especially, the latter dipeptide with the free carboxyl group was obtained easily in a pure form, rather than the methyl ester. Introduction of Z(OMe)-Val-OH by the NP method followed by methylation and subsequent hydrazinolysis gave fragment [18]. Although this fragment has a sterically hindered valine residue at the N-terminus, it did not particularly interfere with the condensation of the subsequent fragment [19].

Scheme 4-c.

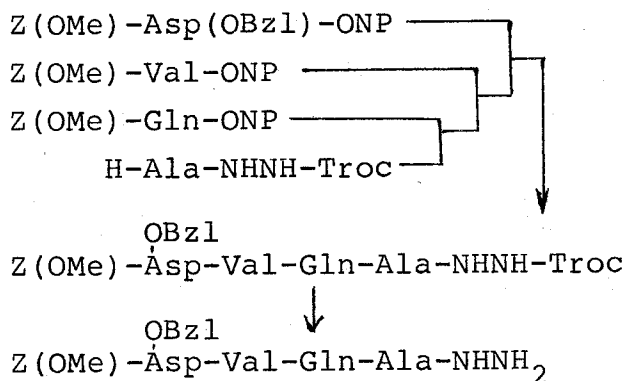
Synthetic scheme of the protected pentapeptide hydrazide, Z(OMe)-(RNase 57-61)-NHNH₂ [18]



The adjacent fragment, Z(OMe)-Asp(OBzl)-Val-Gln-Ala-NHNH-Troc [19] (position 53-56), was prepared with the aid of substituted hydrazine derivative, H-Ala-NHNH-Troc,⁵⁰⁾ as shown in Scheme 4-d. The usual NP method was used to introduce Z(OMe)-Gln-OH, Z(OMe)-Val-OH and Z(OMe)-Asp(OBzl)-OH in a stepwise manner. From the resulting protected tetrapeptide derivative [19], the Troc group was removed with Zn as mentioned above to yield Z(OMe)-Asp(OBzl)-Val-Gln-Ala-NHNH₂.

Scheme 4-d.

Synthetic scheme of the protected tetrapeptide hydrazide derivative, Z(OMe)-(RNase 53-56)-NHNH-Troc [19]

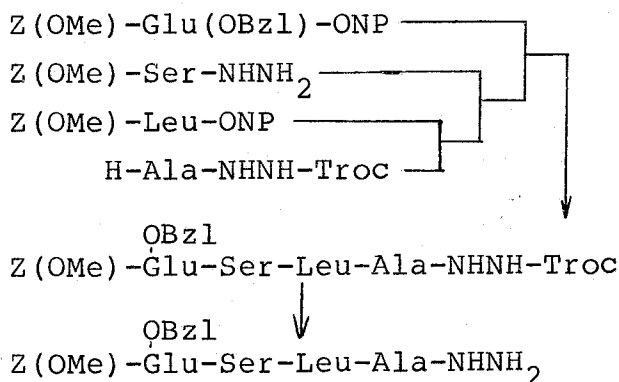


The next fragment, Z(OMe)-Glu(OBzl)-Ser-Leu-Ala-NHNH-Troc [20] (position 49-52), contains Glu(OBzl). This unit was prepared starting with the same substituted hydrazine derivative, H-Ala-NHNH-Troc. As shown in Scheme 4-e, Z(OMe)-Leu-OH and Z(OMe)-Glu(OBzl)-OH were introduced by the NP method and Z(OMe)-Ser-NHNH₂ by the azide procedure. Treatment with Zn removed the Troc group from the resulting tetrapeptide derivative [20] without difficulty to yield Z(OMe)-Glu(OBzl)-Ser-Leu-Ala-NHNH₂.

Z(OMe)-Phe-Val-His-NHNH₂ [21] (position 46-48) was prepared starting with the known dipeptide, Z-Val-His-OMe¹⁰¹⁾ which, after

Scheme 4-e.

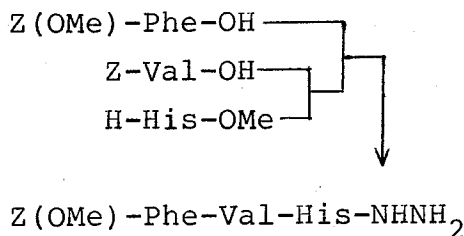
Synthetic scheme of the protected tetrapeptide hydrazide derivative, Z(OMe)-(RNase 49-52)-NHNH-Troc



hydrogenolysis, was condensed with Z(OMe)-Phe-OH by DCC.⁶⁵⁾ The product, after treatment with methanol in the presence of acetic acid for the reason stated earlier,⁶⁷⁾ was converted to the hydrazide [21] as shown in Scheme 4-f.

Scheme 4-f.

Synthetic scheme of the protected tripeptide hydrazide, Z(OMe)-(RNase 46-48)-NHNH₂ [21]



The next fragment, Z(OMe)-Lys(Z)-Pro-Val-Asn-Thr-NHNH₂ [22] (position 41-45), was prepared by the PCP active ester condensation⁷⁶⁾ of Z(OMe)-Lys(Z)-Pro-OH with H-Val-Asn-Thr-OMe. The former active ester is the known compound¹⁰²⁾ and the latter was newly synthesized by two successive NP condensations starting with H-Thr-OMe

solvent succeeded in solubilizing an amino component in an ice-chilled solution, required for the azide reaction.

Poor solubility of synthetic peptide intermediates causes some advantages and disadvantages in the synthesis. Because of great difference in solubilities between larger protected products and smaller activated carboxyl components used in excess during the coupling, the latter could be removed readily by repeated precipitation of the peptide from DMSO with methanol, except in the case of Z(OMe)-(RNase 41-124)-OBzl. The contaminant derived from the fragment [22] was found less soluble in methanol. In this instance, it was possible to purify the desired compound by precipitation from DMSO with DMF, though some compound was sacrificed into a mother liquid.

Despite certain advantages of a relatively insoluble peptide as mentioned above, a serious problem was encountered because it was not possible to find suitable solvent systems for tlc. At the step where the fragment [16] was introduced, the protected peptides and even the N^α-deprotected peptides remained at the origin of the plate in all the solvent systems examined. At this stage, the author lacked a useful tool for the assessment of homogeneity of the peptides, as well as for monitoring the progress of reactions. The expectations for tlc were thus greatly limited. However, at least, it was possible to detect the ninhydrin colour as a guide of coupling reactions and examine the presence or absence of acyl components in isolated products.

The increased insolubility of the larger peptides resulted in a corresponding difficulty in the assessment of the homogeneity of the peptides. Here, the ratios of newly incorporated amino acids in acid hydrolysate to that of phenylalanine gave a very important clue. It is, of course, very important to confirm that observed amino acid ratios are not due to occasional contamination of an unreacted amino component and an acyl component. In this respect,

ratios of phenylalanine and leucine furnish additional information about the homogeneity of the peptides, since one mole of leucine was first introduced with fragment [20]. Combination of tlc and amino acid analysis played a very important role in assessing the purity of the peptides obtained from successive condensations. In addition, the purity of Z(OMe)-(RNase 41-124)-OBzl was checked by gel filtration on Sephacryl S-200. At this stage, it seemed to be suitable to perform such gel-filtration, since some difficulty was observed in removing the acyl component. This place is the 2nd check point of purity by chromatographic means. The synthetic tetraoctacontapeptide ester emerged from a column as a single component. Its amino acid ratios and those of intermediates which were synthesized under conditions mentioned above are listed in Table 4. It is concluded that within the limit of experimental error, these values matched those predicted by theory. Thus, ca. 13 g of Z(OMe)-(RNase 41-124)-OBzl, with a high degree of homogeneity, was synthesized.

Among the intermediates mentioned above, the corresponding sequence 65-124 was taken as an amino component for the condensation with the sequence of 21-64 by Jenkins et al.^{41-c)} Amino acid ratios of Boc-(65-124)-OH were given, but with no diagnostic amino acid, such as phenylalanine.

Table 4. Amino acid ratios of Z(OMe)-(RNase 41-124)-OBzl
and intermediates.

Position Residue	65-124 (60)	62-124 (63)	57-124 (68)	53-124 (72)	49-124 (76)	46-124 (79)	41-124 (84)
Asp	7.12(7)	8.14(8)	8.16(8)	9.22(9)	9.17(9)	9.18(9)	10.20(10)
Thr	5.78(6)	5.74(6)	5.79(6)	5.83(6)	5.89(6)	5.96(6)	6.88 (7)
Ser	4.59(6)	4.79(6)	5.44(7)	5.49(7)	6.43(8)	6.44(8)	6.46 (8)
Glu	5.07(5)	5.16(5)	6.08(6)	7.08(7)	8.10(8)	8.16(8)	8.23 (8)
Pro	2.77(3)	2.79(3)	2.86(3)	2.88(3)	2.93(3)	2.88(3)	3.79 (4)
Gly	3.11(3)	3.12(3)	3.13(3)	3.11(3)	3.21(3)	3.15(3)	3.18 (3)
Ala	3.99(4)	4.89(5)	4.91(5)	5.86(6)	6.88(7)	6.91(7)	6.89 (7)
Val	3.89(4)	4.91(5)	5.89(6)	7.04(7)	6.99(7)	7.92(8)	8.87 (9)
Met	0.66(1)	0.64(1)	0.69(1)	0.71(1)	0.67(1)	0.64(1)	0.73 (1)
Ile	2.41(3)	2.42(3)	2.41(3)	2.49(3)	2.49(3)	2.44(3)	2.47 (3)
Leu					1.01(1)	1.01(1)	1.03 (1)
Tyr	5.30(5)	5.35(5)	5.33(5)	5.31(5)	5.39(5)	5.34(5)	5.37 (5)
Phe	1.00(1)	1.00(1)	1.00(1)	1.00(1)	1.00(1)	2.00(2)	2.00 (2)
Lys	4.27(4)	4.28(4)	5.31(5)	5.26(5)	5.21(5)	5.18(5)	6.21 (6)
His	1.72(2)	1.69(2)	1.72(2)	1.74(2)	1.71(2)	2.76(3)	2.70 (3)
Arg	0.91(1)	0.88(1)	0.86(1)	0.87(1)	0.86(1)	0.89(1)	0.93 (1)
Cys	(5)	(5)	(6)	(6)	(6)	(6)	(6)
Recovery	84%	87%	88%	88%	84%	90%	88%

Section 5. Synthesis of the Protected S-Protein (Positions 21-124) and the Protected S-Peptide (Positions 1-20)

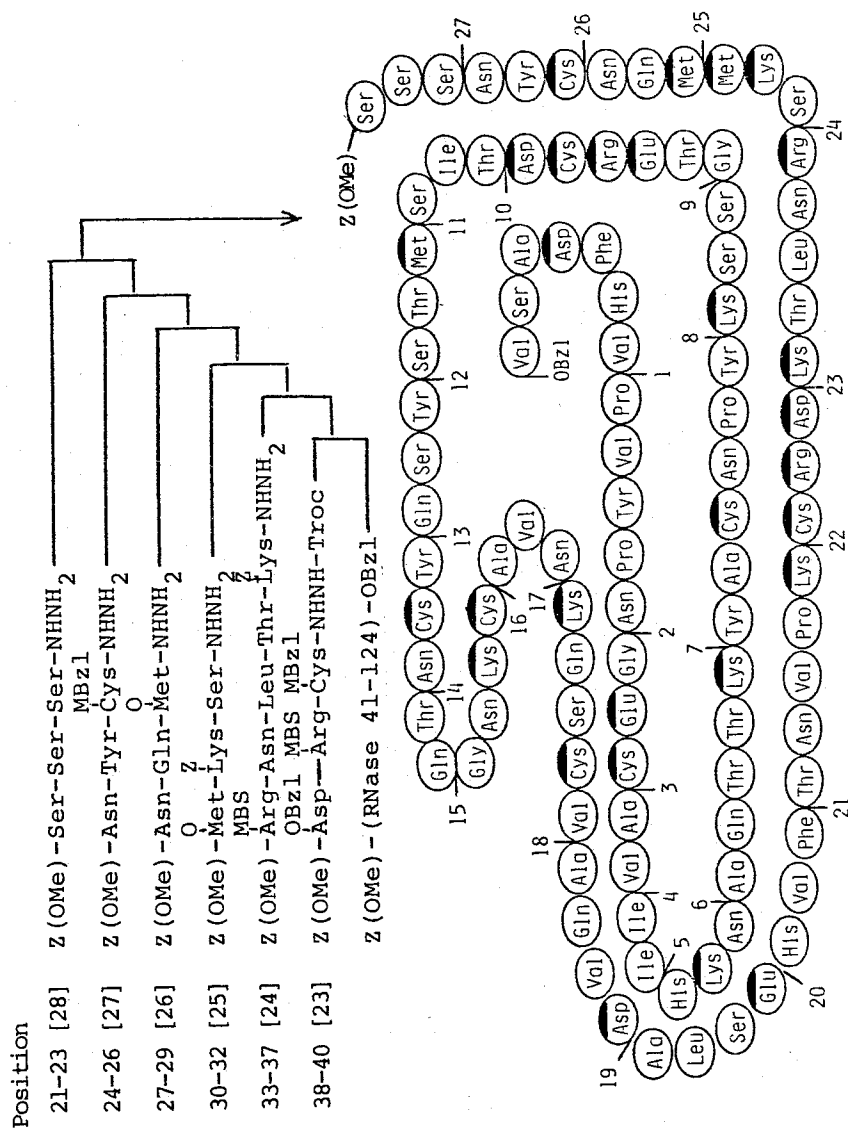
Summary

Starting with the protected tetraoctacontapeptide corresponding to the sequence of bovine pancreatic RNase A, Z(OMe)-(41-124)-OBzl, the protected S-protein, Z(OMe)-(RNase 21-124)-OBzl, was synthesized by successive azide condensation of six peptide fragments; Z(OMe)-Asp(OBzl)-Arg(MBS)-Cys(MBzl)-NHNH-Troc [23], Z(OMe)-Arg(MBS)-Asn-Leu-Thr-Lys(Z)-NHNH₂ [24], Z(OMe)-Met(O)-Lys(Z)-Ser-NHNH₂ [25], Z(OMe)-Asn-Gln-Met(O)-NHNH₂ [26], Z(OMe)-Asn-Tyr-Cys(MBzl)-NHNH₂ [27], Z(OMe)-Ser-Ser-Ser-NHNH₂ [28]. In addition, S-peptide was synthesized in a protected form, Z-Lys(Z)-Glu(OBu^t)-Thr-Ala-Ala-Ala-Lys(Z)-Phe-Glu(OBzl)-Arg(MBS)-Gln-His-Met(O)-Asp-Ser-Ser-Thr-Ser-Ala-Ala-NHNH₂, by assembling three subunits; A(14-20), B (9-13) and C (1-8).

Subsequent to the synthesis of the protected tetraoctacontapeptide ester, Z(OMe)-(RNase 41-124)-OBzl, this peptide chain was elongated to the protected tetraheptapeptide, Z(OMe)-(RNase 21-124)-OBzl, termed the protected S-protein, by successive azide condensation²¹⁾ of relatively small six peptide fragments, as shown in Scheme 5. In addition, the protected eicosapeptide hydrazide, Z-(RNase 1-20)-NHNH₂, termed the protected S-peptide, was synthesized. In this section, a detailed account of experiments involved in these syntheses is described.

It was first planned to construct the N-terminal portion of the protected S-protein by adding fragments, [23], [24], [25] and two additional subunits, Z(OMe)-Ser-Ser-Ser-Asn-Tyr-NHNH₂ (21-25) and Z(OMe)-Cys(MBzl)-Asn-Gln-Met(O)-NHNH₂ (26-29). The preliminary experiments indicated that the Rudinger's azide condensation of the latter proceeded satisfactorily, but for some reasons, the final condensation of the former subunit onto the amino component possessing the Cys(MBzl)-Asn- unit at the N-terminal portion, did not proceed quantitatively, even with the use of a large excess of the acyl component. In the acid hydrolysate of the isolated peptide, recoveries of the newly incorporated amino acids remained at about 76%, indicating that the product was heavily contaminated with the unreacted amino component. If such a product is submitted to subsequent coupling reactions, it is obvious that the product will be contaminated with a failure sequence. At earlier stage of the syntheses, each condensation reaction was driven to completion by use of an excess of acyl component. Removal of the unreacted acyl component was the main consideration in obtaining homogeneous products. In the present situations, the reactivity of the amino components has significantly decreased and removal of unreacted material has become extremely difficult. It is absolutely necessary to drive the reaction as near to completion as possible, even by the use of a larger excesses of acyl components, preferably those

Scheme 5. Synthetic route to the protected tetrahepta peptide ester,
 Z(OMe)-(RNase 21-124)-OBzl (protected S-protein)

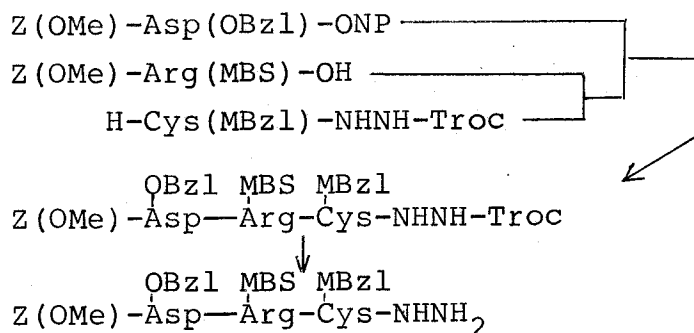


with a higher reactivity. To this end, the above two peptides was subdivided into three subunits; [26], [27] and [28], as shown in Scheme 5.

The necessary fragment, Z(OMe)-Asp(OBzl)-Arg(MBS)-Cys(MBzl)-NHNH-Troc [23] (position 38-40), was prepared in a stepwise manner starting with H-Cys(MBzl)-NHNH-Troc as shown in Scheme 5-a. The DCC plus HOBT¹⁹⁾ and the NP procedures⁶⁴⁾ were employed to introduce Z(OMe)-Arg(MBS)-OH³⁰⁾ and Z(OMe)-Asp(OBzl)-OH respectively. From the resulting protected tripeptide [23], the Troc group⁸⁵⁾ was removed, as usual, by treatment with Zn in acetic acid⁸⁶⁾ to yield Z(OMe)-Asp(OBzl)-Arg(MBS)-Cys(MBzl)-NHNH₂.

Scheme 5-a.

Synthetic scheme of the protected tripeptide hydrazide derivative, Z(OMe)-(RNase 38-40)-NHNH-Troc [23].

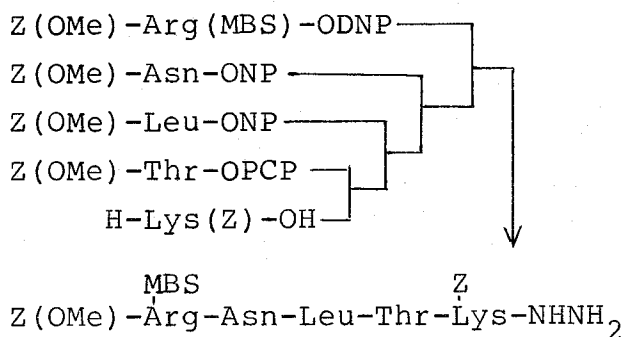


The next fragment, Z(OMe)-Arg(MBS)-Asn-Leu-Thr-Lys(Z)-NHNH₂ [24] (position 33-37), was also prepared in a stepwise manner, but without protection of the C-terminal end. Z(OMe)-Thr-Lys(Z)-OH and Z(OMe)-Leu-Thr-Lys(Z)-OH, prepared by the active ester procedures, respectively, were easily purified by the extraction procedure. Z(OMe)-Asn-OH was then introduced by the NP method, as usual, and Z(OMe)-Arg(MBS)-OH by the DNP method.⁹¹⁾ In the latter instance, the corresponding active ester was not isolated and the progress

of the reaction was monitored by tlc. Thus, possible intramolecular lactam formation¹⁰³⁾ of Arg(MBS) was suppressed. The resulting pentapeptide was esterified and subsequently exposed to hydrazine to give the desired hydrazide as shown in Scheme 5-b.

Scheme 5-b.

Synthetic scheme of the protected pentapeptide hydrazide, Z(OMe)-(RNase 33-37)-NHNH₂ [24].



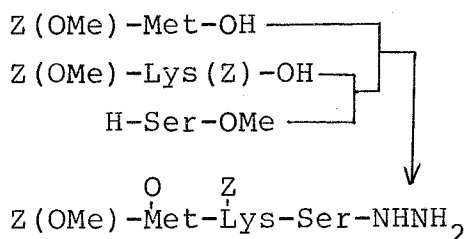
The tripeptide hydrazide, Z(OMe)-Met(O)-Lys(Z)-Ser-NHNH₂ [25] (position 30-32), was prepared according to Scheme 5-c. Z(OMe)-Met-Lys(Z)-Ser-OMe prepared by the DCC condensation⁶⁵⁾ followed by the NP condensation was oxidized by sodium metaperiodate,⁵⁹⁾ instead of hydrogen peroxide³⁷⁾ or tetrachloroauric (II) acid,⁹³⁾ adopted previously for the preparation of Z(OMe)-Ser-Thr-Met(O)-NHNH₂ [12] in Section 3. A convenient procedure for the preparation of Met(O) derivative is now available. This mild oxidation produces a diastereomeric mixture of sulfoxides, but little sulphone.

In order to prepare the fragment, Z(OMe)-Asn-Gln-Met(O)-NHNH₂ [26] (position 27-29), the methionine residue was oxidized by the aforementioned oxidant, sodium metaperiodate, at the dipeptide stage. The resulting dipeptide, Z(OMe)-Gln-Met(O)-OMe was taken to Z(OMe)-Asn-Gln-Met(O)-OMe by the NP method and subsequently to the hydrazide [26] by the usual hydrazine treatment as shown in Scheme

5-d.

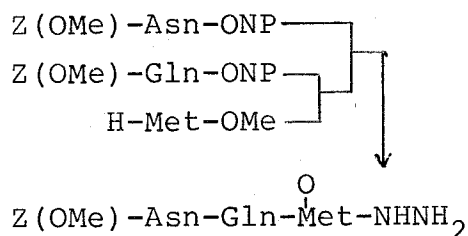
Scheme 5-c.

Synthetic scheme of the protected tripeptide hydrazide, Z(OMe)-(RNase 30-32)-NHNH₂ [25].



Scheme 5-d.

Synthetic scheme of the protected tripeptide hydrazide, Z(OMe)-(RNase 27-29)-NHNH₂ [26].



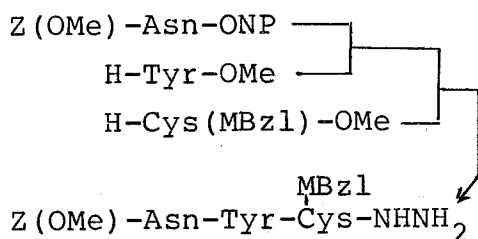
Z(OMe)-Asn-Tyr-Cys(MBzl)-NHNH₂ [27] (position 24-26) is a fragment which was selected for the reason stated earlier. Z(OMe)-Asn-Tyr-NHNH₂ was condensed via the azide with H-Cys(MBzl)-OMe and the resulting tripeptide ester was converted to the hydrazide [27], as usual, as shown in Scheme 5-e.

The N-terminal tripeptide unit, Z(OMe)-Ser-Ser-Ser-NHNH₂ [28] (position 21-23), was prepared by two successive azide condensations followed by the usual hydrazine treatment as shown in Scheme 5-f.

The successive azide condensations of six peptide fragments prepared, as mentioned above, were performed according to Scheme 5. In the azide condensations of [23] and [24], two additions of

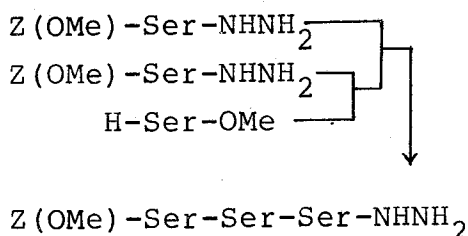
Scheme 5-e.

Synthetic scheme of the protected tripeptide hydrazide, Z(OMe)-(RNase 24-26)-NHNH₂ [27].



Scheme 5-f.

Synthetic scheme of the protected tripeptide hydrazide, Z(OMe)-(RNase 21-23)-NHNH₂ [28].



acyl components (3 + 2 equivalents) in 72 hrs' interval were sufficient to bring the respective reactions to completion, as mentioned in the preceding section. The next condensation of [25] was similarly performed and the reaction mixture became almost ninhydrin negative. When a part of the product was isolated at this stage and submitted to amino acid analysis, incorporation of an acyl component was found to remain at about 80%. Even after purification by repeated washing and precipitation, these situations were not improved. In order to avoid laborious purification problems, after 48 hr, the 3rd shot of an acyl component (2 equivalents) was added to drive the last 20% of the reaction to completion. Based on these experiences, the subsequent condensation of [26], [27] and [28] were performed using 9 to 12 equivalents of an acyl component in each step. Each reaction was started with 5 to 6

equivalents of an acyl component and after 72 hr, the 2nd azide (2 equiv.) was added, with which the reaction mixture became almost ninhydrin negative. In order to secure the complete condensation, the 3rd azide (2 equiv.) was added for the fragment [26] and [27], and the 4th azide (2 equiv.) for the fragment [28]. Amino acid ratios in acid hydrolysates of the protected S-protein and the intermediates which were synthesized under conditions mentioned above are listed in Table 5. These values were judged to match well with those predicted by theory. Phenylalanine, in addition to leucine, still played a very important role as a diagnostic amino acid for assessment of homogeneities. Any R_f values of these compounds could not be recorded because of the unavailability of suitable solvent systems for tlc. In order to obtain additional proof for the homogeneity of synthetic Z(OMe)-(RNase 21-124)-OBzl, the sample was submitted to gel-filtration on Sephacryl S-200. When eluted with DMSO-5% H₂O, the desired compound emerged from the column as a single component, thereby excluding the possibility of contamination by acyl components. (Fig 4).

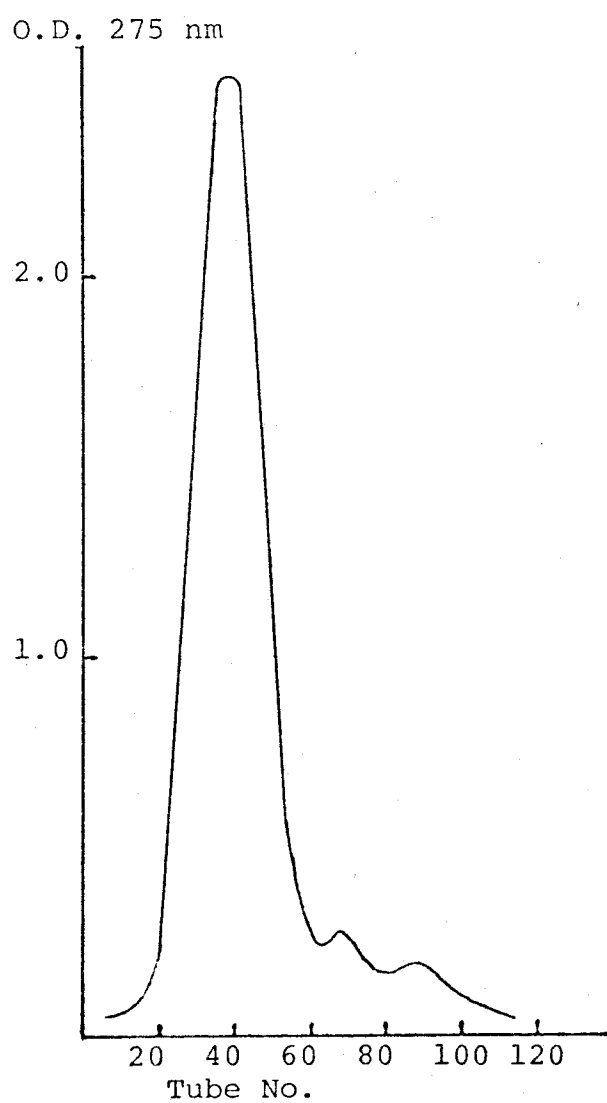
After successive condensations of 28 peptide fragments and column chromatographic examination at three points (positions 69, 41 and 21), 4.9 g of the protected S-protein of a high degree of homogeneity was obtained. Hirschmann et al.^{41-e)} prepared the protected S-protein by the azide condensation of two fragments, Boc-(21-64)-NHNH₂ and H-(65-124)-OH, and this material without purification and characterization, was submitted to the final deprotection. No information about the coupling yield and the purity of the protected S-protein was given.

Table 5. Amino acid ratios of Z(OMe)-(RNase 21-124)-OBzl
and intermediates.

Position Residue	38-124 (87)	33-124 (92)	30-124 (95)	27-124 (98)	24-124 (101)	21-124 (104)
Asp	11.28(11)	12.34(12)	12.34(12)	13.26(13)	14.23(14)	14.20(14)
Thr	6.91 (7)	8.01 (8)	7.96 (8)	7.48 (8)	7.52 (8)	7.69 (8)
Ser	6.52 (8)	6.48 (8)	7.18 (9)	7.29 (9)	7.28 (9)	9.59(12)
Glu	8.21 (8)	8.12 (8)	8.12 (8)	9.44 (9)	9.36 (9)	9.39 (9)
Pro	3.69 (4)	3.79 (4)	3.76 (4)	3.99 (4)	3.77 (4)	3.81 (4)
Gly	3.23 (3)	3.29 (3)	3.17 (3)	3.22 (3)	3.26 (3)	3.02 (3)
Ala	6.81 (7)	6.83 (7)	6.86 (7)	6.91 (7)	6.88 (7)	6.85 (7)
Val	8.79 (9)	8.85 (9)	8.68 (9)	8.65 (9)	8.77 (9)	8.53 (9)
Met*	0.69 (1)	0.70 (1)	1.55 (2)	2.57 (3)	2.37 (3)	2.41 (3)
Ile	2.47 (3)	2.41 (3)	2.38 (3)	2.16 (3)	2.09 (3)	2.01 (3)
Leu	1.01 (1)	2.04 (2)	2.12 (2)	1.91 (2)	1.99 (2)	1.97 (2)
Tyr	5.40 (5)	5.32 (5)	5.29 (5)	5.11 (5)	6.28 (6)	6.26 (6)
Phe	2.00 (2)	2.00 (2)	2.00 (2)	2.00 (2)	2.00 (2)	2.00 (2)
Lys	6.20 (6)	7.34 (7)	8.32 (8)	8.41 (8)	8.36 (8)	8.37 (8)
His	2.71 (3)	2.67 (3)	2.71 (3)	2.31 (3)	2.55 (3)	2.23 (3)
Arg	1.97 (2)	3.05 (3)	2.87 (3)	2.99 (3)	2.81 (3)	2.86 (3)
Cys	(7)	(7)	(7)	(7)	(8)	(8)
Recovery	87%	84%	89%	86%	82%	89%

* Met(O) was not calculated.

Fig.4. Gel-filtration of
Z(OMe)-(RNase 21-124)-OBzl
on Sephacryl S-200



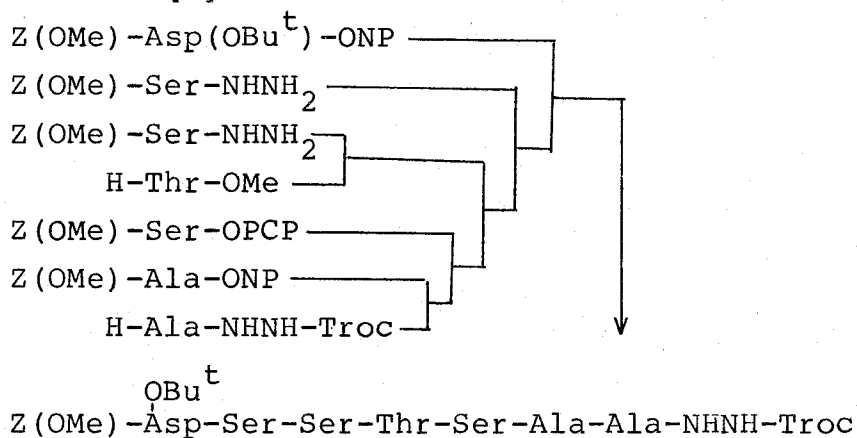
Following the synthesis of the protected S-protein, the author synthesized the S-peptide in a form of the protected hydrazide, expecting to reach the final goal by single azide condensation. Based on the earlier observation made by Richards and his collaborators⁴⁵⁾ that equimolar combination of S-peptide and S-protein brought about full regeneration of enzymic activity (RNase S'), Hofmann et al.¹⁰⁴⁾ synthesized S-peptide in 1966, using protecting groups removable by TFA and claimed the semi-synthesis of RNase S', after combination with natural S-protein. Information about structure-function relationships of S-peptide was presented by the above authors¹⁰⁵⁾ and others.¹⁰⁶⁾

The protected S-peptide hydrazide, Z-(RNase 1-20)-NHNH₂, was synthesized, with the aid of the substituted hydrazine, by the condensation of three building blocks as shown in Scheme 6.

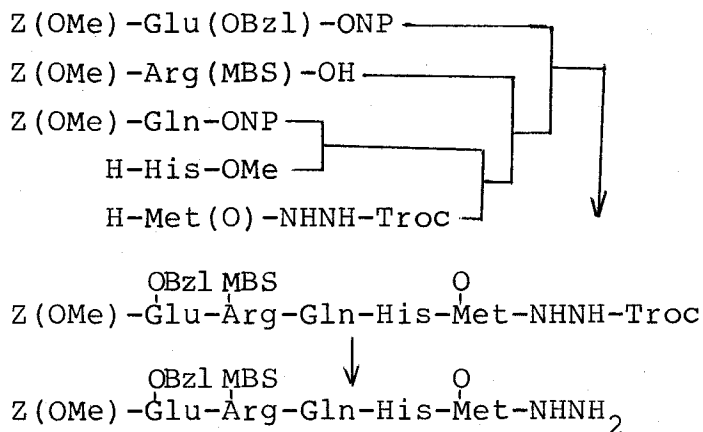
The C-terminal heptapeptide fragment, Z(OMe)-Asp(OBu^t)-Ser-Ser-Thr-Ser-Ala-Ala-NHNH-Troc, Z(OMe)-(RNase 14-20)-NHNH-Troc [A], was prepared according to the Scheme 6-a. Z(OMe)-Ser-Ala-Ala-NHNH-Troc was prepared in a stepwise manner by the active ester procedure, to which Z(OMe)-Ser-Thr-NHNH₂ and Z(OMe)-Ser-NHNH₂ were condensed successively via the azide procedure. Next, Z(OMe)-Asp(OBzl)-OH was added on to the resulting hexapeptide. However, when exposed to Et₃N, the protected heptapeptide gave heterogeneous spots on tlc. This phenomenon seems due to succinimide formation of the Asp(OBzl)-Ser unit as pointed out by Bodanszky et al.¹⁰⁷⁾ Therefore, Z(OMe)-Asp(OBu^t)-OH was instead introduced and both protecting groups was removed by TFA, prior to the next condensation.

The middle fragment, Z(OMe)-Glu(OBzl)-Arg(MBS)-Gln-His-Met(O)-NHNH-Troc, Z(OMe)-(RNase 9-13)-NHNH-Troc [B] containing Glu(OBzl), was synthesized starting with H-Met(O)-NHNH-Troc as shown in Scheme 6-b. Z(OMe)-Gln-His-NHNH₂⁹⁷⁾ was then introduced via the azide, Z(OMe)-Arg(MBS)-OH by the mixed anhydride method⁶⁶⁾ and Z(OMe)-Glu(OBzl)-OH by the NP method respectively. From the resulting

Synthetic scheme of the protected heptapeptide hydrazide derivative, Z(OMe)-(RNase 14-20)-NHNH-Troc [A]



Synthetic scheme of the protected pentapeptide
hydrazide derivative, Z(OMe)-(RNase 9-13)-
NHNH-Troc [B]



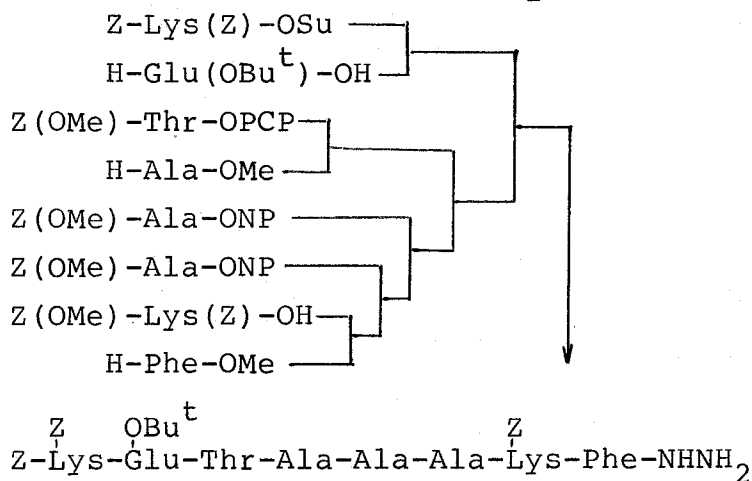
pentapeptide [B], the Troc group was removed with Zn, as usual, to yield Z(OMe)-Glu(OBzl)-Arg(MBS)-Gln-His-Met(O)-NHNH₂.

The N-terminal octapeptide hydrazide, Z-Lys(Z)-Glu(OBu^t)-Thr-Ala-Ala-Ala-Lys(Z)-Phe-NHNH₂, Z-(RNase 1-8)-NHNH₂ [C], contains

the glutamic acid residue. And $\text{Glu}(\text{OBu}^t)$ was applied, instead of $\text{Glu}(\text{OBzl})$, since this ester resists the action of hydrazine¹⁰⁸⁾. As shown in Scheme 6-c, $\text{Z}(\text{OMe})\text{-Ala-Ala-Lys}(\text{Z})\text{-Phe-OMe}$ was prepared in a stepwise manner by either the DCC or NP method and two dipeptide units, $\text{Z}(\text{OMe})\text{-Thr-Ala-NHNH}_2$ and $\text{Z-Lyz}(\text{Z})\text{-Glu}(\text{OBu}^t)\text{-NHNH}_2$, were successively condensed to it: Finally, the resulting octapeptide ester was converted to the corresponding hydrazide in HMPA-MeOH, because of its poor solubility in DMF.

Scheme 6-c.

Synthetic scheme of the protected octapeptide hydrazide, $\text{Z-(RNase 1-8)-NHNH}_2$ [C]



According to Scheme 6, successive azide condensations of three fragments led to the formation of the protected eicosapeptide derivative, from which the Troc group was removed with Zn in a mixture of HMPA-NMP-acetic acid. The Troc group from this relatively large fragment was, for some reason, not removed in DMSO-acetic acid. As mentioned above, the side chain of the aspartic acid residue at position 14 was free in the protected S-peptide. Homogeneity of the protected S-peptide was assessed by tlc, amino acid and elemental analyses. Thus the two peptide units, necessary to construct the

entire amino acid sequence of RNase A, was obtained. The final touch for the total synthesis of RNase is described in the following section.

Section 6. Synthesis of RNase A with the Full Enzymic Activity

Summary

Protected bovine pancreatic RNase A was synthesized by the azide condensation of three fragments; Z(OMe)-(RNase 21-124)-OBzl, Z(OMe)-(RNase 9-20)-NHNH₂ and Z-(RNase 1-8)-NHNH₂.

After removal of the protecting groups by methanesulphonic acid, establishment of the disulphide bridges by air oxidation, and purifications by affinity chromatography and ion-exchange chromatography on CM-cellulose, a synthetic protein was obtained which was indistinguishable by chemical and enzymic criteria from natural RNase A. Comparable results were also obtained, when HF was used as a deprotecting reagent. The unambiguous synthesis of a protein with the full enzymic activity has thus been accomplished for the first time.

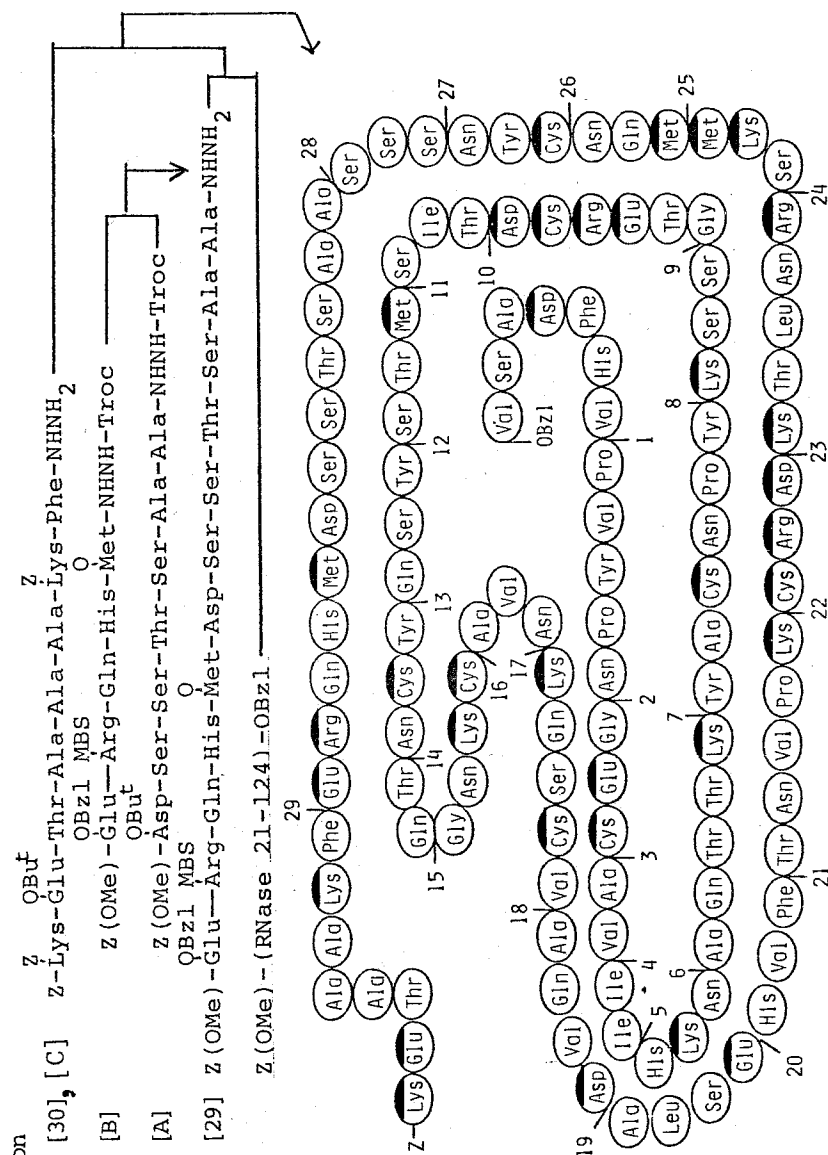
In the preceding section, the syntheses of the protected S-protein, Z(OMe)-(RNase 21-124)-OBzl, and the protected S-peptide, Z-(RNase 1-20)-NHNH₂, which cover the entire amino acid sequence of bovine RNase A have been described. In this section, the synthesis of the protected RNase A, removal of all protecting groups and subsequent establishment of the disulphide bridges are described. A synthetic protein, thus obtained, is indistinguishable from natural bovine RNase A by several chemical criteria, as well as enzymic assay using specific substrates.

As an initial approach to the synthesis of the protected RNase, the azide condensation²¹⁾ of Z-(RNase 1-20)-NHNH₂ and the TFA treated sample of Z(OMe)-(RNase 21-124)-OBzl was performed. In spite of the earlier expectation, this approach encountered great difficulty in driving the reaction to completion. The reaction remained ninhydrin positive, even after two additions of 6 equivalents of the acyl component at 72 hr intervals. Acid hydrolysate of the isolated product revealed the incorporation of the S-peptide portion in only Ca. 22%. The author, therefore, returned to the original strategy, i.e., building up the peptide backbone with small fragments, rather than condensation of the eicosapeptide to the S-protein in one step.

As described in Section 5, the protected S-peptide was synthesized with three fragments, i.e., Z-(RNase 1-8)-NHNH₂ [C], Z(OMe)-(RNase 9-13)-NHNH-Troc [B] and Z(OMe)-(RNase 14-20)-NHNH-Troc [A]. With these available fragments, the most effective route was next examined.

Among possible routes, it does not seem advantageous to submit fragment [A] to condensation with the S-protein unit, since this fragment still carries the base sensitive Asp(OBu^t)-Ser-sequence.^{107,109)} Thus, the following route was judged to be the method of choice: condensation of two subunits [A] and [B] to prepare Z(OMe)-(9-20)-NHNH-Troc and, after removal of the Troc group,^{85,86)}

Scheme 7. Synthetic route to the protected tetracosahexapeptide ester,
Z-(RNase 1-124)-OBzl (protected RNase)



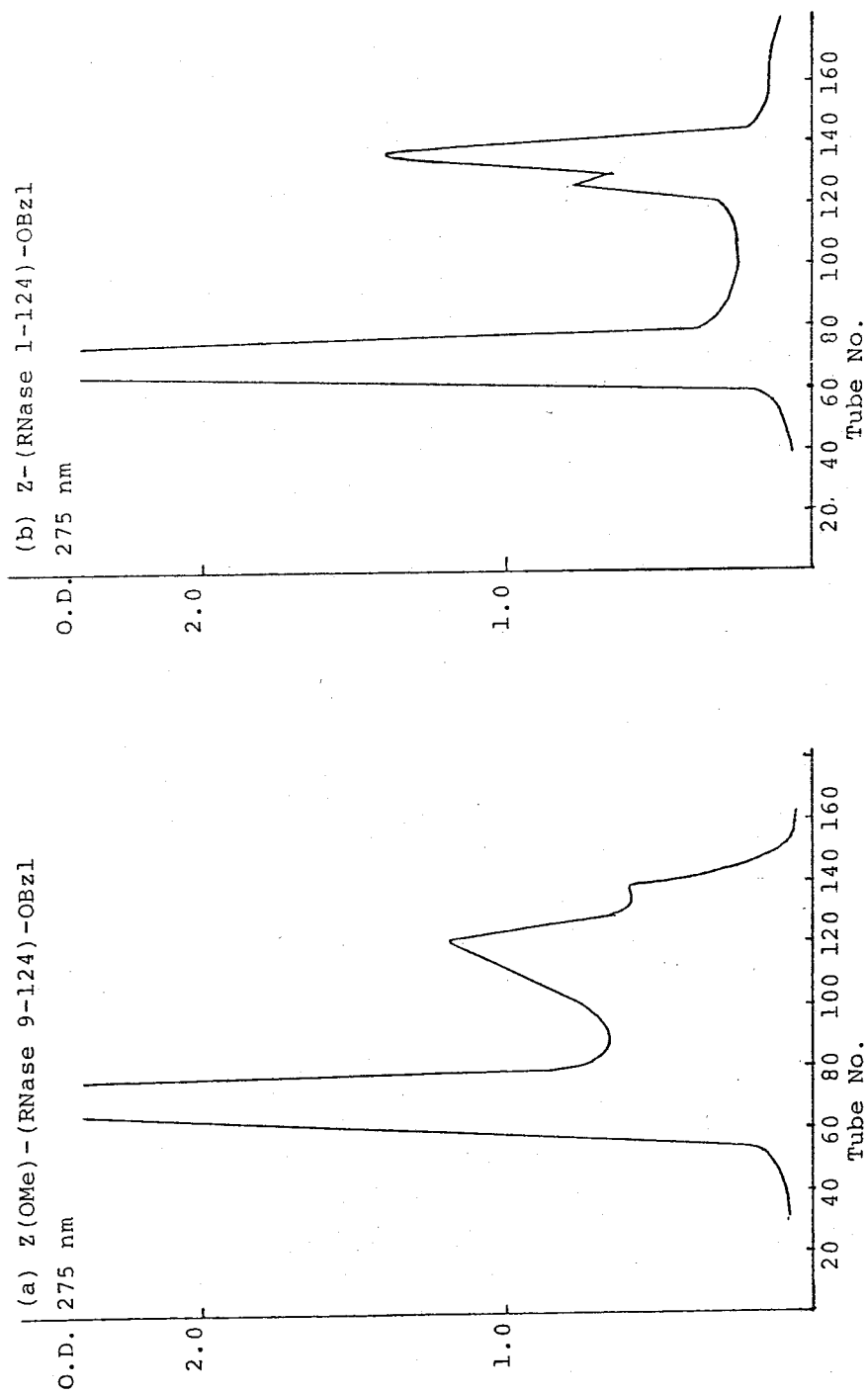
○ Protected amino acid: Asp(OBzl), Glu(OBzl), Cys(MBzl), Lys(Z), Arg(MBS), Met(O).
| Positions of fragment condensation.

condensation with TFA treated S-protein to obtain Z(OMe)-(9-124)-OBzl and condensation of [C] to obtain Z-(1-124)-OBzl, as illustrated in Scheme 7.

Condensation of Z(OMe)-Glu(OBzl)-Arg(MBS)-Gln-His-Met(O)-NHNH₂, derived from [B], with the TFA treated sample of Z(OMe)-Asp(OBu^t)-Ser-Ser-Thr-Ser-Ala-Ala-NHNH-Troc [A] proceeded smoothly as described in Section 5 to produce the protected dodecapeptide, Z(OMe)-Glu(OBzl)-Arg(MBS)-Gln-His-Met(O)-Asp-Ser-Ser-Thr-Ser-Ala-Ala-NHNH-Troc, from which the Troc group was removed by Zn.^{85,86} The resulting dodecapeptide hydrazide, Z(OMe)-Glu(OBzl)-Arg(MBS)-Gln-His-Met(O)-Asp-Ser-Ser-Thr-Ser-Ala-Ala-NHNH₂ [29] (position 9-20) was selected as one fragment for the synthesis of RNase. As mentioned in Section 5, the β -carboxyl protecting group of Asp was removed during the TFA treatment to suppress $\alpha\rightarrow\beta$ rearrangement in the following reactions.

Chain elongation of the S-protein unit was next carried out according to Scheme 7. The Z(OMe) group was removed from Z(OMe)-(21-124)-OBzl by treatment with TFA⁶¹ and the N ^{α} -deprotected peptide was dissolved in a solvent mixture, (DMSO-HMPA). As mentioned in the preceding section, no single solvent dissolved the N ^{α} -deprotected peptide. After neutralization with Et₃N, this amino component was submitted to condensation with 10 equivalents of the acyl component derived from Z(OMe)-(RNase 9-20)-NHNH₂. After 72 hr, an additional 10 equivalents of the acyl component was added. After an additional 48 hr, the reaction mixture became almost ninhydrin negative, when tested by a tlc scanner. In order to drive the coupling reaction to completion, two times of 5 equivalents of the azide were further added at successive 24 hr intervals. After 7 days, the product was isolated by precipitation from DMSO by addition of MeOH and purified by gel-filtration on Sephacryl S-200 with DMSO-H₂O (95:5) as eluate (Fig. 5-a). The unreacted acyl component used in excess was easily separated from the desired product. Tlc exam-

Fig. 5. Purification of Z(OMe)-(RNase 9-124)-OBzl (a) and Z-(RNase 1-124)-OBzl (b) by gel-filtration on Sephacryl S-200



ination of the product was still unsuccessful, since it stayed at the origin on the plate with all solvent systems examined. However, no contamination of the acyl component was found by tlc. Amino acid ratios in the acid hydrolysate revealed the incorporation of the acyl component in nearly 100%, when the recovery of Phe or Leu (2 residue each) was taken as a standard of calculation. Next, Z(OMe)-(9-124)-OBzl, thus obtained, was submitted to the final condensation with Z-(RNase 1-8)-NHNH₂ [30],[C]. The condensation reaction was performed in essentially the same manner as mentioned above with a total of 30 equivalents of the acyl component. The product was purified by gel-filtration on Sephadryl S-200 with DMSO-H₂O (95:5) as eluate (Fig. 5-b). The unreacted acyl component was clearly separated from the desired protected RNase.

In this final condensation, one residue of Phe was incorporated into the chain. Amino acid composition of the protected RNase could also be calculated by taking either the recovery of Leu or that of Phe as a standard. Two moles of Leu were incorporated into the peptide chain at position 51 and 35 and the ratios of Leu(2) and Phe(2) were in excellent agreement with each other, prior to final condensation. Their ratios, Leu(2)/Phe(3), were now found to be 1.97/3.00. The coupling efficiency in the final step could be judged to be quantitative (Table 6).

Starting with 1.6 g of the protected S-protein, 0.98 g of the protected RNase with a high degree of homogeneity was obtained. Within the 30 successive fragment condensations performed in the present synthesis, the most laborious step was the condensation of a relatively large fragment [29] with the N^α-deprotected S-protein. Each of the 30 fragments and their 97 intermediates were fully characterized by elemental analysis. Fragment condensations were performed at least three times in each step to obtain enough materials. Products obtained were all examined by amino acid analysis. Consequently, it is emphasized that the present synthesis of the

Table 6.
Amino acid ratios of synthetic RNase and intermediates

	Protected peptides		Air oxi- Affinity purified CM-puri- Natural					
	9-124	1-124	Reduced	dized	Peak 1	Peak 2	fied	RNase
Asp	15.36(15)	15.34(15)	14.84	16.17	13.75	15.54	15.06	14.99(15)
Thr	8.52 (9)	9.39(10)	9.00	8.99	8.84	9.89	9.90	9.97(10)
Ser	12.17(15)	11.93(15)	11.66	10.88	12.63	14.61	13.95	14.37(15)
Glu	11.67(11)	12.64(12)	12.08	12.27	12.05	12.59	12.48	12.44(12)
Pro	3.78 (4)	4.11 (4)	4.10	3.52	4.24	4.43	4.35	4.43 (4)
Gly	3.09 (3)	3.09 (3)	3.02	3.17	3.27	3.73	3.31	3.40 (3)
Ala	8.89 (9)	12.11(12)	11.84	11.65	10.81	11.90	12.59	12.16(12)
Cys	3.11 (4)	2.82 (4)	3.50	2.27	2.20	3.25	3.65	3.72 (4)
Val	8.71 (9)	8.71 (9)	8.53	8.71	8.00	8.81	9.25	8.88 (9)
Met	3.33 (4)	3.31 (4)	3.68	1.75	3.00	3.08	3.65	3.85 (4)
Ile	1.94 (3)	1.99 (3)	2.13	1.96	1.84	2.08	2.35	2.31 (3)
Leu	2.00 (2)	2.00 (2)	2.00	2.00	2.00	2.00	2.00	2.00 (2)
Tyr	6.32 (6)	6.19 (6)	6.06	2.49	2.98	5.65	5.82	5.66 (6)
Phe	2.03 (2)	3.04 (3)	2.91	2.37	1.87	2.59	3.03	3.09 (3)
Lys	8.37 (8)	10.57(10)	10.17	11.97	8.88	10.85	10.67	10.82(10)
His	3.20 (4)	3.36 (4)	3.24	3.11	2.80	3.29	3.93	4.03 (4)
Arg	3.92 (4)	3.87 (4)	3.68	4.10	3.45	4.13	4.00	4.20 (4)
Recovery	88%	85%	95%	70%	73%	77%	79%	80%

protected RNase was supported by the accumulation of considerable chemical evidences as described in Section 1-6.

As stated in Section 1, deprotection with methanesulphonic acid (MSA)²²⁾ was the initial strategy for the present synthesis of RNase. Prior to the deprotection, the first problem was the recovery of cysteine from Cys(MBzl) by this acid. In the presence of various cation scavengers, Z(OMe)-Cys(MBzl)-OH was treated with MSA, as well as HF²⁵⁾ for comparison and the treated samples were examined by amino acid analyses. When the sample was treated with MSA in the presence of anisole or m-cresol, nearly quantitative recovery of cysteine was obtained, however the treated sample in the presence of thioanisole or other sulphur compounds gave several unidentified peaks and recovery of cysteine remained less than 75%. Thus it became apparent that cation scavengers play an important role for the satisfactory recovery of cysteine from Cys(MBzl) in the MSA deprotecting procedure. It seems worthwhile to mention that the HF-anisole deprotection gave some side products, but a satisfactory result was obtained when the HF-m-cresol system was employed. Considering also the recent investigation of the scavenger system reported by Lundt et al.,¹¹⁰⁾ as will be mentioned later, it was decided to try two methods of deprotection, i.e., MSA and HF with m-cresol as a cation scavenger.

In this connection, the next problem was the occasional appearance of an unidentified peak on the short column of an amino acid analyser with a retention time of 28 minutes, when acid hydrolyses of protected peptides containing Cys(MBzl) are performed in the presence of phenol.¹¹¹⁾ The synthetic protected RNase was found to give this unidentified peak under these conditions. Through model experiments, this compound was identified as p-hydroxyphenyl-cysteine³⁸⁾ derived from the sulfoxide of Cys(MBzl) in the 6N HCl-phenol system. The identical product was obtained when the sulfoxide of Z(OMe)-Cys(MBzl)-OH was exposed to MSA, as well as HF,

in the presence of phenol. When this treatment was performed in the presence of anisole, p-methoxyphenylcysteine was obtained in both acidolytic deprotections. Z(OMe)-Cys(MBzl)-OH with no sulfoxide gave no such side product, even in the presence of phenol or anisole.

These results suggested that the Cys(MBzl) residue of the synthetic protected RNase were partially oxidized to the corresponding sulfoxide during the synthesis and satisfactory recovery of cysteine could never be expected, unless reduced prior to deprotection. The use of thiophenol as a reducing agent overcame this difficulty. However, under this condition, Met(O) residues^{37,38)} were also reduced back to Met, thus removing its protective property from attack by carbonium cations derived from exposing the Z, Bzl, or MBzl group with MSA. Fortunately, the S-alkylated compounds if formed, can be removed by incubation with thiol compounds.¹¹²⁾ Therefore, it seemed that, in addition to the reduction of the sulfoxide of Cys(MBzl) prior to deprotection, selection of m-cresol as a scavenger, instead of anisole, was one of the elements, which guided the synthesis of RNase to a successful completion.

As shown in Chapter 2- Section 3, in the MSA-anisole system, the methyl group of anisole is transformed to Met to form S-methyl-Met sulphonium salt,³¹⁾ however, the MSA-cresol has no property as an alkyl donor. Furthermore, m-cresol is more efficient than anisole in suppressing not only the side reaction of Cys(MBzl) mentioned above, but also another side reaction involving the modification of Tyr residue^{57,58)} during the MSA and HF deprotection, i.e., partial O-sulphonylation of Tyr from Arg(MBS) and the partial intermolecular alkylation at the 3 position by attack of carbonyl cations derived from the protecting groups. The intramolecular alkylation from Tyr(Bzl) is a well known serious side reaction.²⁸⁾ However, the present synthesis is free of the latter problem, since unprotected Tyr was used.

As was pointed out, m-cresol suppresses most side reactions which may occur during the MSA deprotection. In addition, in deprotection with acid, one has to take accounts of the N \rightarrow O shift at the Ser and Thr residues. The side reaction was mentioned in the HF deprotection^{113,114)} and also pointed out in the MSA deprotection by Fujino et al.¹¹⁵⁾ A mild base treatment is known to be effective to reverse this rearrangement. Another possible side reaction is the $\alpha \rightarrow \beta$ rearrangement Asp(OBzl) or Asp(OBu^t).^{107,109)} And the protecting group was removed from Asp at position 14 adjacent to Ser, since this residue is known to cause side reactions of this type. Though, Suzuki et al.¹¹⁶⁾ mentioned little tendency in the MSA deprotection, complete prevention of such side reaction seems impossible.

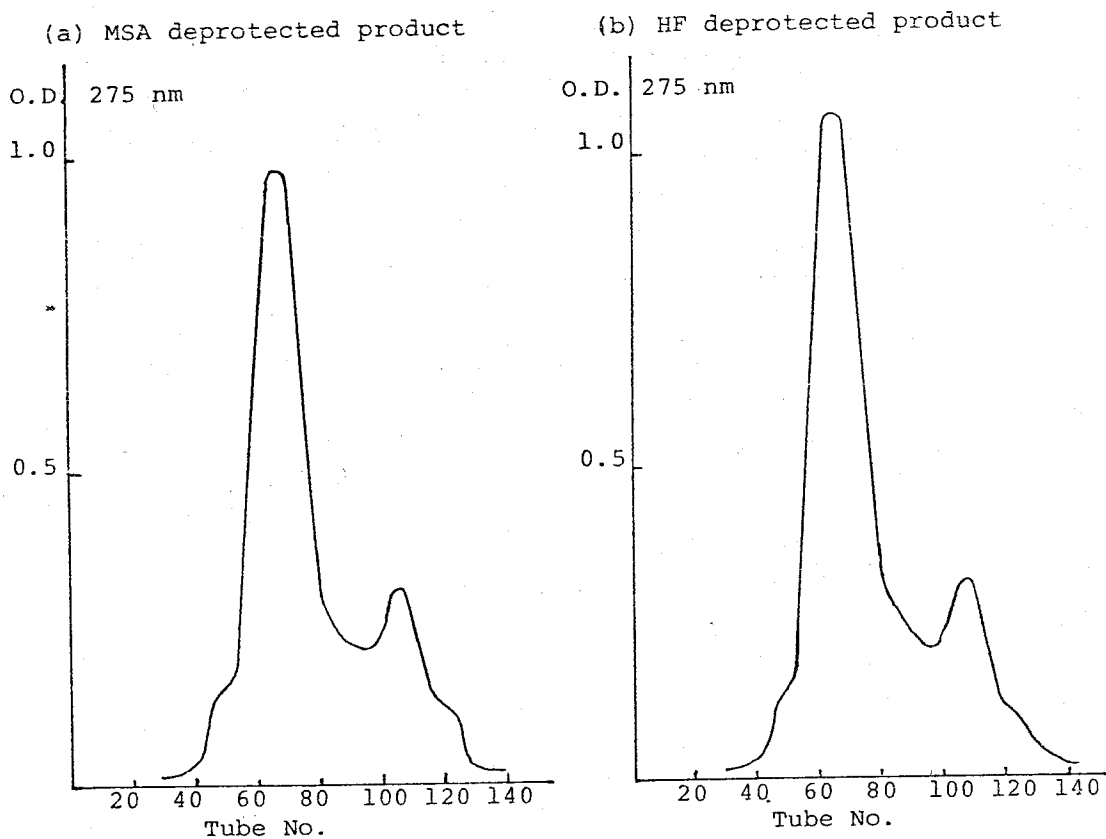
The protected RNase was treated with thiophenol to reduce the partially formed sulfoxide of Cys(MBzl), using Z(OMe)-Cys(MBzl)(O)-OH and Z(OMe)-Met(O)-OH as the markers. The Met(O) residues were also reduced under these conditions. First, the MSA deprotection is described.

Under a nitrogen gas atmosphere, the reduced peptide was exposed to MSA in the presence of m-cresol at 25° for 60 minutes. The amount of the scavenger was estimated as 20 equivalents for each protecting group (33 protecting groups). Thus a total of 660 equivalents were employed. In order to remove many protecting groups, i.e., 8 x Cys(MBzl), 4 x Glu(OBzl), 1 x Glu(OBu^t), 4 x Asp(OBzl), 4 x Arg(MBS), 10 x Lys(Z) and the N- and C-terminal protecting groups, Z and Bzl, this treatment was repeated. Somewhat drastic conditions seemed necessary to completely remove so many protecting groups, otherwise it is apparent that one would have a complicated mixture of partially deprotected peptides. The deprotected peptide was then reduced with 2-mercaptoethanol and dithiothreitol in 0.2M Tris-HCl buffer containing 4M guanidine HCl at pH 8.6. This pH value was the one selected by Anfinsen and

Haber⁴⁸⁾ for reductive cleavage of the disulphides of natural bovine RNase. This basic condition seemed suitable to reverse any possible $N \rightarrow O$ shift at the Ser and Thr residues. This treatment was also effective for reduction of remaining Met(O), if present, and for removal of the S-alkyl groups from the Met residues, if such products were formed during the deprotection. And aggregated disulphide complex, if formed, could then be reduced during this treatment.

The reduced product was next isolated from the reducing reagent by gel-filtration on Sephadex G-25 with 0.1N AcOH as eluate. The reduced product emerged from the column as a single component and the eluates of the desired fractions were then diluted in 0.05 M Tris-HCl buffer at pH 8.2 to a concentration of 0.02 mg/ml. The dilute solution was kept standing at 25° for 2 days to establish the 4 intramolecular disulphide bridges.^{48,117)} The progress of the oxidation was monitored by Ellman's reagent.¹¹⁸⁾ The entire solution was concentrated by lyophilization and the oxidized product was then dialyzed against distilled water for desalting. Some water-insoluble material, presumably highly aggregated products or materials still bearing protecting groups, was removed at this stage. The oxidized product was then isolated by gel-filtration on Sephadex G-75 with 0.05M NH_4HCO_3 as eluent (Fig. 6-a). The oxidized product, thus obtained, was found to have 9-12% of the activity of natural RNase on yeast RNA.¹¹⁹⁾ Starting with the protected RNase, the yield of this crude, but active product was 54%. Gutte and Merrifield⁹⁾ attempted, in the first run, to purify the deprotected peptide through the corresponding S-sulphonate. The preliminary test indicated that the oxidative sulphitolysis¹²⁰⁾ necessary to prepare the S-sulphonate converted Met to Met(O) in 10%. Thus, it was felt that this procedure would be effective to purify Cys-peptides which contain no Met, such as insulin,⁷⁾ but not applicable to the present synthesis.

Fig. 6. Purification of the air oxidized product by gel-filtration on Sephadex G-75



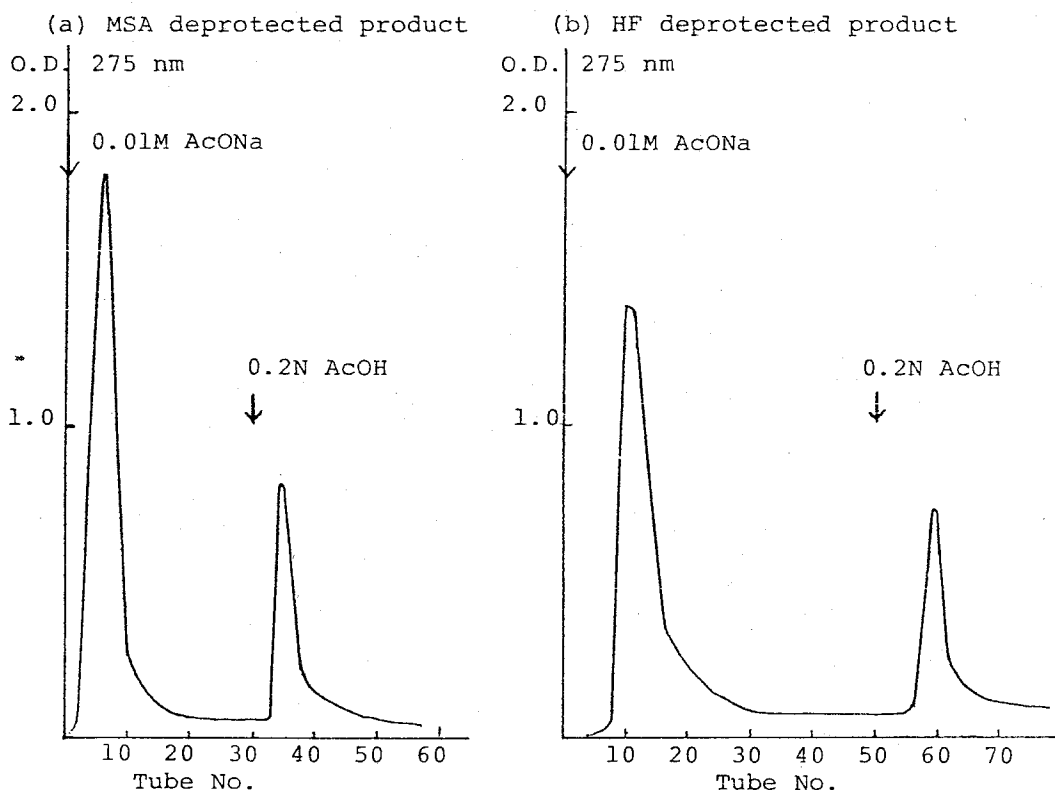
Next, it was decided to purify the crude, active product by affinity chromatography on Sepharose-(4B)-5'-(4-aminophenylphosphoryl)-uridine-2'(3')-phosphate according to Wilchek and Gorecki.⁴⁹⁾ This purification procedure worked nicely (Fig. 7-a). The material isolated from the fractions which passed through the column without retention was inactive. The product eluted with 0.2N AcOH exhibited an activity of 74 to 82% against yeast RNA, when examined at three different concentrations.

The yield of this purified step was 14%. Thus, to reach this point, approximately 92% of the material was sacrificed during the conversion of the inactive protected peptide to the enzymatically active product. Various side reactions involved in the deprotection and the miss-leading disulphide bridge formation may be the main reasons responsible for lowering the yield.

Disc electrophoretic examination of the active component at pH 4.3 revealed the presence of a single band, matched with that of natural RNase and an adjacent, faint band. When amino acid compositions in the acid hydrolysate of active and inactive compounds were compared, no significant differences were observed, except for recoveries of Tyr and Cys (Table 6). Recoveries of these amino acids in the active component were much closer to those of natural RNase, compared to those of the inactive component. The activity at this stage is nearly equivalent to that reported by Gutte and Merrifield.⁹⁾ They reported that the supernatant of the ammonium sulphate fractionation of the trypsin-resistant material exhibited an activity of 78%. This solution, which was estimated to contain 0.41 mg of a protein, was claimed as the most active component they obtained. However, this fraction was not chemically characterized.

In order to remove the trace contaminant revealed by disc-electrophoresis, the affinity purified sample was submitted to ion-exchange chromatography on CM-cellulose.^{117-a)} The chromatogra-

Fig. 7. Purification of the G-75 purified product by affinity chromatography on agarose-5'-(4-aminophenyl-phosphoryl)-uridine-2'(3')-phosphate



phic pattern eluted by a gradient with pH 7.5, 0.1M sodium phosphate buffer revealed the presence of a major peak with a small shoulder to the fore (Fig 8-a). The small front fractions were discarded. The main fractions were desalted by gel-filtration on Sephadex G-25 and the product was examined by disc electrophoresis and amino acid analysis. The purified product behaved as a single component in the field of disc electrophoresis at pH 4.3 and its mobility was identical with that of natural RNase. The amino acid compositions of the synthetic protein was finally in excellent agreement with that of natural RNase, including those of Tyr and Cys residues (Table 6). The synthetic protein with high degree of homogeneity exhibited essentially the same activity as that of natural RNase, when yeast RNA was used as a substrate and measured at three different concentrations. The values which obtained were 104-107%.

Next, the physicochemical properties and biological activities of the synthetic protein were examined. It was first confirmed that the elution pattern of the synthetic protein from the CM-cellulose column was comparable to that of natural RNase. In addition to the disc-electrophoretic mobilities of both compounds, it was confirmed that the specific rotation, UV absorption and Michaelis constant of the synthetic protein were coincident with those of natural RNase.^{117-a, 121)}

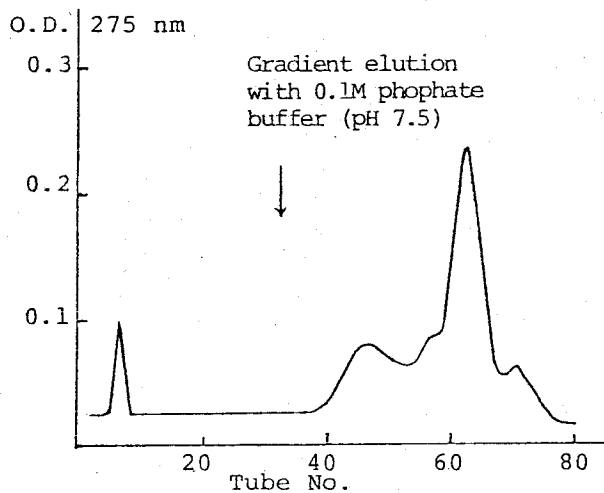
In order to examine further the specific enzymatic activity of the synthetic protein, the activity against 2',3'-cyclic cytidine phosphate¹²²⁾ was examined. Results comparable to that of natural RNase were obtained in this assay. The synthetic protein had no ability to digest calf thymus DNA.¹²³⁾ Thus, the specific activity of the synthetic enzyme was ascertained by three different assay systems.

In order to confirm the results obtained from the MSA deprotection, HF deprotection was conducted in the presence of m-cresol

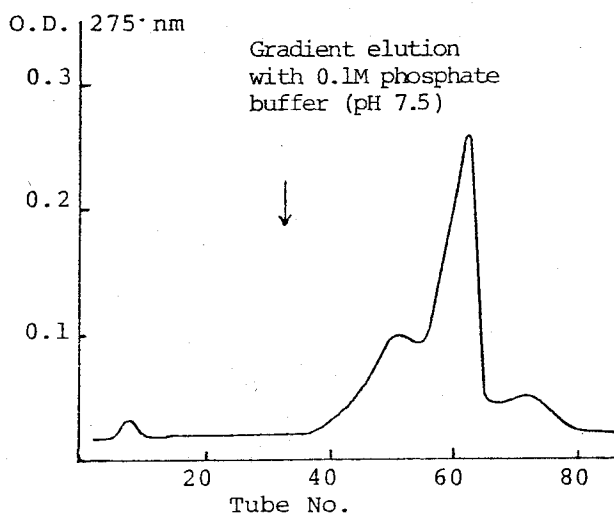
Fig. 8.

Purification of the affinity purified product by ion-exchange chromatography on CM-cellulose

(a) MSA deprotected peptide



(b) HF deprotected peptide



and the product was treated in the same manner as mentioned above. The crude air-oxidized product isolated by gel-filtration on Sephadex G-75 (Fig. 6-b) possessed a slightly higher activity (17%) than that of the MSA deprotected product, though the amount of water-insoluble material was somewhat greater than in the former instance. The affinity purified product (Fig. 7-b) was as active as that of the MSA deprotected product (82%). After purification on CM-cellulose (Fig. 8-b), the fully active component was obtained in nearly the same yield.

From the experimental data cited above, it can be ascertained that the synthetic protein has a high degree of homogeneity and possesses physicochemical properties and specific enzymatic activity comparable to those of natural RNase. It is concluded that the unambiguous synthesis of a protein with full activity has thus been accomplished for the first time.

Summary

Bovine pancreatic ribonuclease A which consists of 124 amino acids was synthesized by conventional method using the methane-sulphonic acid deprotecting procedure at the final stage. Synthetic protein was indistinguishable from natural ribonuclease A by several chemical criteria (electrophoretic mobility, ultraviolet spectra, specific rotation) as well as enzymic assay using specific substrates for ribonuclease A (yeast RNA, 2',3'-cyclic cytidine phosphate).

This is the first synthesis of the protein with the full enzymic activity of ribonuclease A.

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EXPERIMENTAL SECTION

Experimental Section

Chapter 2. Examination of Deprotecting Reagents for Peptide Synthesis

Section 1. Trifluoromethanesulphonic Acid, as a Deprotecting Reagent in Peptide Chemistry

GENERAL PROCEDURE

Treatment of Amino Acid derivatives with TFMSA. —————

Each amino acid derivative (1 mmol) was dissolved at 20° in methylene chloride or TFA (9.2 ml) containing anisole (1.5-3 equiv.) and TFMSA (5-10 equiv.) was added. A part of the solution was subjected to quantitative amino acid analysis. The results were listed in the Text.

Section 2. Methanesulphonic Acid, as a Deprotecting Reagent in Peptide Chemistry

GENERAL PROCEDURE

Treatment of Amino Acid Derivatives with Sulphonic Acids. —————

Treatment of amino acid derivatives (1 mmol) with bromocamphor sulphonic acids (10 equiv.) was performed in the presence of anisole (1.5-5equiv.) at 20° for 30 min. As a solvent, TFA (9.2 ml) was employed. Treatment of amino acid derivatives with MSA (100 equiv.) was performed in essentially the same manner as described above, except for that no solvent was employed. The results were listed in the Text.

Section 3. Examination of Methanesulphonic Acid Procedure for the Synthesis of Peptides Containing Methionine

GENERAL PROCEDURE

Chemical shifts in ^{13}C -nuclear magnetic resonance (CMR) were measured with respect to internal dioxane and converted to the Me_4Si scale using the formula $\delta_{\text{Me}_4\text{Si}} = \delta_{\text{(dioxane)}} + 67.4 \text{ ppm}$.

Tlc was performed on silica gel (Kieselgel G, Merck) and R_f value refers to the following solvent system. R_{f1} n-BuOH-AcOH-Pyridine- H_2O (4:1:1:2).

S-Methylmethionine Dimethanesulphonate.

(a) Treatment of Met. — A mixture of Met (0.5 g), anisole (1 ml, 2.8 equiv.) and MSA (3 ml, 14 equiv.) was kept on standing at room temperature (22°). When monitored by tlc, Met (R_{f1} 0.48) disappeared completely within 24 hr and a new single spot (R_{f1} 0.1) was detected. Dry ether was added and the resulting oily precipitate was washed three times with ether. Treatment of the residue with EtOH afforded a crystalline compound, which was recrystallized from EtOH; yield 851 mg (72%), m.p. $196\text{--}201^\circ$, $[\alpha]_D^{24} + 16.6^\circ$ (c 1.0 in 5% AcOH). IR $\nu_{\text{max}} \text{ cm}^{-1}$ 1715 (CO), 3400 (NH). Proton magnetic resonance (PMR): (D_2O): δ 2.80 (6H, s., Me), 2.97 (6H, s., Me), 3.89 (1H, t., $\alpha\text{-CH}$), 2.40 (2H, m., CH_2), 3.48 (2H, m., CH_2). CMR (dioxane): δ 170.9 (CO), 51.9 (CH), 39.9 (CH_2), 39.4 (CH_2), 25.5 (Me), 25.2 (Me). (Found: C, 27.19; H, 5.88; N, 3.97; S, 26.76. $\text{C}_6\text{H}_{14}\text{NO}_2\text{S}\cdot\text{CH}_3\text{SO}_3\cdot\text{CH}_3\text{SO}_3\text{H}$ requires C, 27.03; H, 5.96; N, 3.94; S, 27.06%). This compound emerged from the short column of an amino acid analyser between His and ammonia peaks (retention time 27 min.) and can be converted to homoserine by treatment with 1N NaOH at 80° for 3 hr.

(b) Treatment of Z-Met-OH. — A mixture of Z-Met-OH (540 mg), anisole (1 ml) and MSA (3 ml) was kept on standing at room temperature for 24 hr. The product, m.p. $192\text{--}197^\circ$, isolated similarly was

and identical with the methanesulphonate obtained in (a) by comparison with their IR spectra; yield 467 mg (69%). Isolation of the benzylsulphonium salt was unsuccessful and its formation could not be detected by tlc.

Preparation from S-Methylmethionine Iodide.—To a solution of methylmethionine iodide (200 mg) in H_2O (2 ml) was added a saturated solution of picric acid to give a yellow precipitate, which was washed with a small amount of H_2O and recrystallized from 50% aqueous EtOH; yield 264 mg (62%), m.p. 154–156°. (Found: C, 34.81; 2.79; N, 15.73; S, 5.23. $C_6H_{14}NO_2S \cdot C_6H_2N_3O_7 \cdot C_6H_3N_3O_7$ requires 34.79; H, 3.08; N, 15.78; S, 5.16%).

The above dipicrate (510 mg) was dissolved in 30% aqueous MSA solution, after washing with ether until the ether layer became colorless, was concentrated to dryness under reduced pressure to give the dimethanesulphonate (I), which was recrystallized from EtOH; yield 231 mg (79%), m.p. 199–201°. (Found: C, 27.12; H, 6.11; S, 3.69%).

Treatment of Met with TFMSA.—A mixture of Met (504 mg) and TFA (1 ml) in 33% TFMSA-TFA (3 ml) was kept at room temperature 24 hr. Dry ether was added to form an oily precipitate, which was washed with ether and then dissolved in a small amount of H_2O . A saturated aqueous solution of picric acid was added and the resulting precipitate was crystallized from 50% aqueous EtOH; yield 231 mg (58%), m.p. 154–156°. Its IR spectra were identical with those of the authentic dipicrate obtained above.

Ethylmethionine Sulphonium Bromide Salt—(1.50 g) was treated with MSA (15 ml) in the presence of phenethylamine (1.5 ml) at room temperature overnight. Dry ether was added and the resulting oily precipitate was dissolved in H_2O (50 ml). The solution was applied to a column of Amberlite CG-120 (H^+ form, 10 cm), which, after washing with H_2O (100 ml), was eluted with 1N

NH_4OH . The latter eluates, after addition of 47% HBr (1 ml), was passed through a column of Amberlite IRA-410 (acetate form, 2 x 10 cm). Eluates were then lyophilized and the residue was treated with EtOH to give a solid; yield 0.90 g (35%), $[\alpha]_D^{26} + 23.3^\circ$ (c 1.0 in AcOH), (Found: C, 32.98; H, 6.07; N, 5.47; S, 12.89; Br, 31.71. $\text{C}_7\text{H}_{16}\text{BrNO}_2\text{S}$ requires C, 32.56; H, 6.24; N, 5.42; S, 12.47; Br, 30.95%).

Identification of Phenol from Anisole

A mixture of anisole (1.08 g) and Met (2.24 g, 1.5 equiv.) in MSA (5.8 ml, 9 equiv.) was kept at room temperature overnight and n-hexane and ice were added. The organic phase was washed with 1% NaOH. The washing was acidified with 6N HCl, saturated with NaCl and extracted with ether. The ether extract was dried over Na_2SO_4 and concentrated to dryness to give needles; yield 0.55 g (59%). The product was identified as phenol by comparison of their IR spectra and gas chromatographic examination.

Chapter 3. Total Synthesis of Bovine Pancreatic Ribonuclease A

General Procedures and Materials

Melting points are uncorrected. Rotations were determined with a Union digital polarimeter PM-101. Hydrolyses of protected peptides containing Tyr with 6N HCl were performed in the presence of phenol (ca. 20 equiv.).¹¹¹⁾ The amino acid compositions of acid hydrolysates were determined with a Hitachi amino acid analyser Model KLA-5 and values are uncorrected for amino acid destruction. Solvents were freshly distilled and evaporations were carried out in vacuo at a bath temperature of 40-50°.

The N^α-protecting group, Z(OMe), was cleaved by TFA in the presence of anisole (2 equiv. or more) in an ice-bath for 45-60 min.

The DCC and the active ester condensations were performed at room temperature (17-25°).

The azide condensation was performed according to Honzl and Rudinger²¹⁾; To an ice-chilled solution of a hydrazide in DMF, HCl-DMF (2 equiv.) was added, followed by isoamyl nitrite (1.1 equiv.). After stirring for 15 to 20 min, when the hydrazine test¹²⁴⁾ became negative, the solution was neutralized with Et₃N (2 equiv.) and added to an ice-chilled solution of an amino component, together with additional Et₃N (1 equiv.). In the azide reaction derived from C-terminal His-hydrazides, N-methylmorpholine, instead of Et₃N, was employed for suppression of possible racemization.¹²⁵⁾ After stirring at 4° for 48 hr (in most instances), a few drops of AcOH was added and the solution was concentrated. In the later stage of the synthesis a large excess of the azide component was employed by subdividing into 3 to 4 portions at certain intervals. For measurement of a small amount of isoamyl nitrite, Et₃N and N-methylmorpholine, Pipetman P200 (Gilson CO.) was used.

A mixed anhydride was prepared according to Vaughan and

Osato⁶⁶⁾: Isobutyl chloroformate (1.1 equiv.) was added to an ice-chilled solution of a carboxyl component and Et_3N (1 equiv.) in dry THF. After stirring for 20 min, the solution was added to an ice-chilled solution of an amino component in DMF.

Unless otherwise mentioning, products were purified by either one of the following procedures:

Procedure A: For purification of protected peptide esters soluble in AcOEt, the extract was washed with 5% citric acid, 5% NaHCO_3 and H_2O -NaCl, dried over Na_2SO_4 and concentrated. The residue was recrystallized or precipitated from appropriate solvents.

Procedure B: For purification of protected peptide esters less soluble in AcOEt, the crude product was triturated with ether and 5% citric acid. The resulting powder was washed with 5% citric acid, 5% NaHCO_3 and H_2O , and recrystallized or precipitated from appropriate solvents. For purification of His-containing peptides, 3% AcOH, instead of 5% citric acid, was used for washing.

Procedure C: For purification of protected peptides with the free carboxyl group, the crude product was dissolved in 3% ammonia and the aqueous phase, after washing with AcOEt, was acidified with citric acid. The resulting powder (if an oil was obtained, it was extracted with AcOEt) was washed with 5% citric acid and H_2O and recrystallized or precipitated from appropriate solvents.

Thin layer chromatography (tlc) was performed on Silicagel (Kieselgel G, Merck). Rf values refer to the following solvent systems; Rf_1 CHCl_3 -MeOH- H_2O (8:3:1), Rf_2 CHCl_3 -MeOH-AcOH (9:1:0.5), Rf_3 n-BuOH-AcOH-AcOEt- H_2O (1:1:1:1), Rf_4 n-BuOH-AcOH-Pyr- H_2O (4:1:1:2), Rf_5 n-BuOH-AcOH- H_2O (4:1:5), Rf_6 CHCl_3 - $\text{CF}_3\text{CH}_2\text{OH}$ (3:1).

For dialysis, cellulose tubing, VT351 (Lot. No. V9H1874), was purchased from Nakarai Chemical Co.. For column chromatography, a fraction collector, Dainihonseiki Model DNS-DFC-100, was used. UV absorption was measured by a Hitachi spectrophotometer, Model 200-20. Hitachi wavelength tunable effluent monitor (034-0029) was used

determine the enzymic activity against 2',3'-cyclic cytidine sphate. For affinity chromatography, Sepharose (4B)-5'-(4-aminophosphoryl)-uridine-2'(3')-phosphate was prepared according Wilchek and Gorecki.⁴⁹⁾ The following enzymes and substrates were chased; natural bovine pancreatic RNase (Type 1-A, Lot. No. 47c-2), 2',3'-cyclic cytidine monophosphoric acid sodium salt (Lot. 76c-7510), bovine pancreatic DNase I (Lot. No. 127c-0347) and st RNA (Type XI, Lot. No. 124c-8510) from Sigma Chemical Co., f thymus DNA (Lot. No. 38N687P) from Worthington Biochemical Co.

Section 1. Synthesis of the Protected Pentadecapeptide
Ester (Positions 110-124)

Z(OMe)-Ala-Ser-OMe ————— DCC (41.20 g, 0.2 mol)
was added to a stirred mixture of Z(OMe)-Ala-OH (43.05g, 0.17 mol) and H-Ser-OMe (prepared from 26.45 g, 0.17 mol of the hydrochloride with 23.5 ml, 0.17 mol of Et₃N) in DMF (300 ml). After 48 hr, the solution was filtered, the filtrate was concentrated and the residue was purified by procedure A. Recrystallization from AcOEt gave the ester (40.01 g, 66%), m.p. 143-145°, $[\alpha]_D^{22} + 6.3^\circ$ (c 0.6 in DMF), Rf₁ 0.81 (Found: C, 54.47; H, 6.32; N, 7.94. C₁₆H₂₂N₂O₇ requires C, 54.23; H, 6.26; N, 7.91%).

* Z(OMe)-Ala-Ser-NHNH₂ ————— To a solution of Z(OMe)-Ala-Ser-OMe (25.0 g, 71 mmol) in MeOH (250 ml) was added 80% hydrazine hydrate (25 ml, 0.4 mol). The precipitate formed on standing at room temperature over night was collected by filtration and recrystallized from MeOH to give the crystalline hydrazide (24.07 g, 96%), m.p. 210-211°, $[\alpha]_D^{22} + 15.5^\circ$ (c 0.6 in DMSO), Rf₁ 0.72 (Found: C, 51.04; H, 6.29; N, 15.56. C₁₅H₂₂N₄O₆ requires C, 50.84; H, 6.26; N, 15.81%).

Z(OMe)-Ala-Ser-Val-OBzl ————— The azide (prepared from 17.70 g, 50 mmol of Z(OMe)-Ala-Ser-NHNH₂) in DMF (85 ml) and Et₃N 6.9 ml, 50 mmol) were added to a stirred solution of H-Val-OBzl (prepared from 14.41 g, 50 mmol of the hydrobromide with 6.9 ml, 50 mmol of Et₃N) in DMF (85 ml) and, after the reaction, the solution was concentrated. The residue was purified by procedure A followed by recrystallization from MeOH to afford the crystalline tripeptide ester (16.33 g, 62%), m.p. 146-151°. $[\alpha]_D^{22} - 29.3^\circ$ (c 0.6 in MeOH), Rf₁ 0.68 (Found: C, 61.19; H, 6.70; N, 8.09. C₂₇H₃₅N₃O₈ requires C, 61.23; H, 6.66; N, 7.94%).

Z(OMe)-Asp(OBzl)-Ala-Ser-Val-OBzl ————— Z(OMe)-Ala-Ser-Val-OBzl (23.71 g, 45 mmol) was treated with TFA-anisole (30 ml-12 ml) usual and n-hexane was added to form an oily precipitate, which was washed with ether, dried over KOH pellets in vacuo for 3 hr and dissolved in DMF (200 ml), together with Et₃N (12.4 ml, 90 mmol) and Z(OMe)-Asp(OBzl)-ONP (29.49 g, 58 mmol). After stirring for 48 hr the solution was concentrated. The product was purified by procedure A and further by column chromatography on silica (3 x 35 cm), which was eluted with CHCl₃-MeOH (8:2, V/V). Fractions containing the substance of Rf₁ 0.98 were combined and the solvent was evaporated. The residue was recrystallized from AcOEt to give the protected tetrapeptide ester (26.79 g, 81%), m.p. 129-131°, $[\alpha]_D^{22}$ - 12.1° (c 1.2 in DMF), (Found: C, 62.17; H, 6.34; N, 7.59. H₄₆N₄O₁₁ requires C, 62.11; H, 6.31; N, 7.62%).

Z(OMe)-Phe-Asp(OBzl)-Ala-Ser-Val-OBzl. ————— Z(OMe)-(OBzl)-Ala-Ser-Val-OBzl (7.34 g, 10 mmol) was treated with TFA-anisole (7 ml-3 ml) and n-hexane was added. An oily residue was washed with n-hexane and dissolved in 3.15N HCl-dioxane (3.1 ml, 10 mmol). After evaporation of the solvent, the residue was washed with n-hexane, dried over KOH pellets in vacuo for 3 hr and then dissolved in DMF (70 ml) together with Et₃N (1.4 ml, 10 mmol), Z(OMe)-Phe-OH (3.96 g, 12 mmol) and DCC (2.47 g, 12 mmol). After stirring for 48 hr, the solution was filtered, the filtrate was concentrated and the residue was purified by procedure B followed by recrystallization from THF and AcOEt to give a white powder (5.5 g, 97%), m.p. 203-206°. $[\alpha]_D^{22}$ -13.9° (c 0.7 in DMF), Rf₂ 0.80 (Found: C, 63.20; H, 6.31; N, 7.91. C₄₇H₅₅N₅O_{12.1/2} H₂O: C, 63.35; H, 6.34; N, 7.86%).

Z(OMe)-Val-His-OMe ————— A mixed anhydride (prepared from 45.01 g, 0.16 mol of Z(OMe)-Val-OH) in THF (250 ml) was

added to an ice-chilled solution of H-His-OMe (prepared from 38.56 g, 0.16 mol of the dihydrochloride with 44.2 ml, 0.32 mol of Et_3N) in DMF (200 ml). After stirring in an ice-bath for 5 hr, the solution was concentrated and the residue was triturated with ether. Batchwise washing with 5% Na_2CO_3 and H_2O followed by recrystallization from MeOH and ether afforded the crystalline ester (40.03 g, 58%), m.p. 166-167°, $[\alpha]_{\text{D}}^{22} + 4.1^\circ$ (c 0.7 in DMF), Rf_1 0.54 (Found: C, 58.22; H, 6.68; N, 12.58. $\text{C}_{21}\text{H}_{28}\text{N}_4\text{O}_6$ requires C, 58.32; H, 6.53; N, 12.96%).

$\text{Z(OMe)-Val-His-NHNH}_2$ ————— $\text{Z(OMe)-Val-His-OMe}$ (40.02 g, 92 mmol) dissolved in MeOH (200 ml) was treated with 80% hydrazine hydrate (25.7 ml, 0.41 mol) at room temperature overnight. The resulting solid was collected by filtration and washed with MeOH to give a powder (27.50 g, 69%), m.p. 160-161°. $[\alpha]_{\text{D}}^{25} - 10.8^\circ$ (c 0.8 in DMSO), Rf_1 0.47 (Found: C, 54.79; H, 6.66; N, 19.31. $\text{C}_{20}\text{H}_{28}\text{N}_6\text{O}_5 \cdot 1/2 \text{H}_2\text{O}$ requires C, 54.41; H, 6.62; N, 19.04%).

$\text{Z(OMe)-Val-His-Phe-Asp(OBzl)-Ala-Ser-Val-OBzl}$, $\text{Z(OMe)-(RNase 118-124)-OBzl}$ [1]. ————— $\text{Z(OMe)-Phe-Asp(OBzl)-Ala-Ser-Val-OBzl}$ (8.82 g, 10 mmol) was treated with TFA-anisole (12 ml-4 ml) as usual and n-hexane was added. An oily residue was washed with n-hexane, dried over KOH pellets in vacuo for 3 hr and then dissolved in DMF (20 ml) containing Et_3N (1.4 ml, 10 mmol). To this ice-chilled solution, the azide (prepared from 5.56 g, 13 mmol of $\text{Z(OMe)-Val-His-NHNH}_2$) in DMF (40 ml) and N-methylmorpholine (1.4 ml, 13 mmol) were added. After the reaction, the solution was concentrated and the residue was treated with ether and 5% NaHCO_3 . The resulting powder was purified by column chromatography on silica (3 x 35 cm), which was eluted with CHCl_3 -MeOH- H_2O (8:3:1 v/v). Fractions containing the substance of Rf_1 0.72 were combined and the solvent was evaporated. The residue was triturated with

and then recrystallized from THF and ether to give the protect-
 heptapeptide ester as a powder (8.72 g, 78%), m.p. 190-193°, $[\alpha]_D^{25} - 18.9^\circ$ (c 1.0 in DMF), Rf_1 0.72 (Found C, 62.41; H, 6.41; N, 11.06. $C_{58}H_{71}N_9O_{14}$ requires C, 62.29; H, 6.40; N, 11.27%).

Z(OMe)-Pro-Tyr-Val-OMe. ————— The azide (prepared from 10 g, 55 mmol of Z(OMe)-Pro-Tyr-NHNH₂⁶⁹) in DMF (100 ml) and Et₃N (7.6 ml, 55 mmol) were added to an ice-chilled solution of Val-OMe (prepared from 11.20 g, 67 mmol of the hydrochloride and 9.2 ml, 67 mmol of Et₃N) in DMF (50 ml). After the reaction, the solution was concentrated and the residue was purified by procedure A. Trituration with ether and recrystallization twice from Et₂O afforded the tripeptide ester as a powder (29.42 g, 96%), m.p. 166-169°, $[\alpha]_D^{16} - 49.7^\circ$ (c 0.9 in DMF), Rf_2 0.63 (Found: C, 62.63; H, 6.70; N, 7.51. $C_{29}H_{37}N_3O_8$ requires C, 62.69; H, 6.71; N, 7.52%).

Z(OMe)-Pro-Tyr-Val-NHNH₂. ————— Z(OMe)-Pro-Tyr-Val-OMe (10 g, 53 mmol) dissolved in MeOH (200 ml) was treated with 80% hydrazine hydrate (63 ml, 1.0 mol) at 60° for 8 hr. After cooling to ice, the resulting solid was collected by filtration and recrystallized twice from DMF with MeOH to afford the tripeptide hydrazide (22.50 g, 73%), m.p. 253-255°, $[\alpha]_D^{16} - 27.7^\circ$ (c 0.8 in DMSO), Rf_1 0.40 (Found: C, 60.75; H, 6.79; N, 12.50. $C_{28}H_{37}N_5O_7$ requires C, 60.52; H, 6.71; N, 12.61%).

Z(OMe)-Pro-Tyr-Val-Pro-OH. ————— The azide (prepared from 10 g, 41 mmol of Z(OMe)-Pro-Tyr-Val-NHNH₂) in DMF (100 ml) and Et₃N (5.6 ml, 41 mmol) were added to an ice-chilled solution of Val-Pro-OH (7.01 g, 61 mmol) in H₂O (30 ml) containing Et₃N (8.4 ml, 61 mmol). After stirring for 24 hr, the mixture was concentrated and the residue was purified by procedure C. Trituration with ether

and recrystallization twice from AcOEt and ether afforded a powder (20.66 g, 80%), m.p. 137-141°, $[\alpha]_D^{25} - 57.0^\circ$ (c 0.8 in DMF), Rf_2 0.26 (Found: C, 61.55; H, 6.94; N, 8.48. $C_{33}H_{42}N_4O_9 \cdot 1/2 H_2O$ requires C, 61.19; H, 6.69; N, 8.65%).

Z(OMe)-Asn-Pro-Tyr-Val-Pro-OH, Z(OMe)-(RNase 113-117)-OH [2].

—— Z(OMe)-Pro-Tyr-Val-Pro-OH (17.30 g, 26 mmol) was treated with TFA-anisole (35 ml-10 ml) as usual and dry ether was added. The resulting powder was dried over KOH pellets in vacuo for 3 hr and dissolved in DMF (100 ml) together with Et_3N (8.0 ml, 58 mmol), HOBT (6.10 g, 29 mmol) and Z(OMe)-Asn-ONP (12.10 g, 29 mmol). After stirring for 24 hr, the mixture was concentrated and the residue was purified by procedure C followed by recrystallization twice from MeOH and AcOEt to give the protected pentapeptide (14.54 g, 72%), m.p. 144-148°, $[\alpha]_D^{25} - 74.5^\circ$ (c 0.8 in DMF), Rf_1 0.38, Rf_2 0.20. Amino acid analysis (Numbers in parentheses are theory): Asp 1.00(1), Pro 2.11(2), Tyr 0.96(1), Val 0.93(1), average recovery 90% (Found: C, 57.89; H, 6.42; N, 10.72. $C_{37}H_{48}N_6O_{11} \cdot H_2O$ requires C, 57.65; H, 6.54; N, 10.90%).

Z(OMe)-Cys(MBzl)-Glu(OBzl)-Gly-OH, Z(OMe)-(RNase 110-112)-OH

[3]. —— Z(OMe)-Glu(OBzl)-Gly-OH⁶⁹ (22.60 g, 50 mmol) was treated with TFA-anisole (24 ml-12 ml) as usual and dry ether was added. An oily precipitate was dried over KOH pellets in vacuo for 3 hr and dissolved in DMF (200 ml) together with Et_3N (14 ml, 0.1 mol) and Z(OMe)-Cys (MBzl)-ONP (31.57 g, 60 mmol). After stirring for 48 hr, the mixture was concentrated and the residue was treated with 5% citric acid and ether. The resulting powder was recrystallized twice from AcOEt and ether to give the protected tripeptide (23.80 g, 70%), m.p. 124-126°. $[\alpha]_D^{22} - 50.3^\circ$ (c 0.8 in DMF), Rf_1 0.42. Amino acid analysis: Glu 1.07(1), Gly 1.00(1), average recovery 91% (Found: C, 59.68; H, 5.81; N, 6.00. $C_{34}H_{39}N_3O_{10}S$ re-

quires C, 59.90; H, 5.77; N, 6.16%).

Z(OMe)-Asn-Pro-Tyr-Val-Pro-Val-His-Phe-Asp(OBzl)-Ala-Ser-Val-OBzl, Z(OMe)-(RNase 113-124)-OBzl. ————— To a solution of Z(OMe)-Asn-Pro-Tyr-Val-Pro-OH (25.0 g, 32 mmol) in DMF (150 ml) was added Et₃N (4.4 ml, 32 mmol) followed by PCP-O-TCA (14.41 g, 35 mmol). While stirring for 2 hr, a new spot of Rf₂ 0.32 appeared on tlc and the starting material of Rf₂ 0.20 disappeared. The solvent was evaporated and the residue was triturated with H₂O and the resulting active ester was recrystallized from THF and ether as a powder (27.55 g, 86%), m.p. 136-141°. Z(OMe)-Val-His-Phe-Asp(OBzl)-Ala-Ser-Val-OBzl (23.52 g, 21 mmol) was treated with TFA-anisole (70 ml-15 ml) as usual and dry ether was added. The resulting powder was dried over KOH pellets in vacuo for 3 hr and then dissolved in DMF (150 ml) together with Et₃N (9.6 ml, 69 mmol), HOBT (4.83 g, 23 mmol) and the active ester obtained above (23.20 g, 23 mmol). After stirring for 48 hr, the solution was concentrated and the residue was purified by procedure B followed by precipitation from MeOH with AcOEt to give a powder (31.47 g, 89%), m.p. 148-151°. $[\alpha]_D^{22}$ -50.3° (c 0.8 in DMF), Rf₁ 0.60, Rf₃ 0.76 (Found: C, 59.99; H, 6.56; N, 12.27. C₈₆H₁₀₉N₁₅O₂₁·2H₂O requires C, 59.98; H, 6.60; N, 12.18%).

Z(OMe)-Cys(MBzl)-Glu(OBzl)-Gly-Asn-Pro-Tyr-Val-Pro-Val-His-Phe-Asp(OBzl)-Ala-Ser-Val-OBzl, Z(OMe)-(RNase 110-124)-OBzl. ————— PCP-O-TCA (9.49 g, 23 mmol) was added to a solution of Z(OMe)-Cys(MBzl)-Glu(OBzl)-Gly-OH (14.20 g, 21 mmol) in DMF (70 ml) containing Et₃N (3.15 ml, 23 mmol). While stirring for 2.5 hr, the starting material (Rf₁ 0.42) disappeared and a new spot (Rf₁ 0.92) was detected on tlc. The solvent was evaporated and the residue was treated with H₂O and the resulting powder was precipitated from THF with ether to afford the active ester (15.96 g, 82%). Z(OMe)-

(RNase 113-124)-OBzl (20.30 g, 12 mmol) was treated with TFA-anisole (60 ml-15 ml) and the deprotected peptide, isolated as mentioned above, was dissolved in DMF (100 ml) together with Et₃N (5.46 ml, 40 mmol), HOBT (2.71 g, 13 mmol) and the active ester obtained above (12.10 g, 13 mmol). After stirring for 48 hr, the mixture was concentrated and the residue was purified by procedure B followed by precipitation from DMF with MeOH to give the protected pentadecapeptide ester as a powder (18.02 g, 70%), m.p. 226-231°, $[\alpha]_D^{22}$ - 51.2° (c 0.9 in DMF), Rf₁ 0.58 (Found: C, 60.04; H, 6.47; N, 11.37; S, 1.63. C₁₁₁H₁₃₈N₁₈O₂₇·2H₂O requires C, 59.93; H, 6.43; N, 11.34; S, 1.44%).

Trial Experiments

Z-Ala-Ser-Val-OH. ——— The azide (prepared from 19.20 g, 60 mmol of Z-Ala-Ser-NHNH₂)⁶³⁾ in DMF (150 ml) and Et₃N (8.3 ml, 60 mmol) were added to a solution of Val (13.92 g, 0.12 mol) in H₂O (130 ml) and pyridine (70 ml) containing Et₃N (16.6 ml, 0.12 mol). While stirring for 48 hr, a certain amount of Val separated from the solution. The solution was filtered and the filtrate was concentrated. The residue was purified by procedure C followed by recrystallization from AcOEt to give the protected tripeptide (7.85 g, 32%), m.p. 165-166°, $[\alpha]_D^{22}$ +5.2° (c 1.0 in DMF), Rf₁ 0.15 (Found: C, 55.98; H, 6.89; N, 10.23; C₁₉H₂₇N₃O₇ required C, 55.73; H, 6.65; N, 10.26%).

H-Ala-Ser-Val-OH. ——— Z-Ala-Ser-Val-OH (13.10 g, 32 mmol) in MeOH (250 ml) containing a few drops of AcOH was hydrogenated over a Pd catalyst for 8 hr. The solution was filtered, the filtrate was concentrated and the residue, after trituration with EtOH, was recrystallized from H₂O and EtOH to give a crystalline compound (8.60 g, 98%), m.p. 210° dec., $[\alpha]_D^{22}$ - 39.1° (c 1.0 in 3% AcOH), Rf₄ 0.41 (Found: C, 48.10; H, 7.82; N, 15.06. C₁₁H₂₁N₃O₅

requires C, 47.99; H, 7.69; N, 15.27%).

Z-Pro-Val-His-OMe. ——— Z-Val-His-OMe¹²⁶ (40.20 g, 0.1 mol) dissolved in MeOH (300 ml) and 1N HCl (200 ml, 0.2 mol) was hydrogenated over a Pd catalyst for 8 hr. The catalyst was removed by filtration, the filtrate was concentrated and the residue (Rf₃ 0.48) was dissolved in acetonitrile (400 ml) together with Et₃N (28 ml, 0.2 mol) and Z-Pro-OH (24.90 g, 0.1 mol). DCC (24.80 g, 0.12 mol) was added and the mixture was stirred overnight. The solution was filtered, the filtrate was concentrated and the residue was dissolved in AcOEt. The organic phase was washed with 5% Na₂CO₃ and H₂O-NaCl, dried over Na₂SO₄ and then concentrated. The residue was recrystallized from MeOH and ether to give the protected tripeptide ester (28.11 g, 56%), m.p. 131-135°, $[\alpha]_D^{22} - 28.7^\circ$ (c 0.9 in DMF), Rf₁ 0.66 (Found: C, 58.33; H, 6.95; N, 13.66. C₂₅H₃₃N₅O₆·H₂O requires C, 58.01; H, 6.82; N, 13.52%).

Z(OMe)-Val-Pro-Val-His-OMe. ——— Z-Pro-Val-His-OMe (26.0 g, 50 mmol) in MeOH (300 ml) and 1N HCl (110 ml, 0.11 mol) was hydrogenated over a Pd catalyst for 8 hr. After filtration, the solution was concentrated and the residue was dissolved in DMF (100 ml) together with Et₃N (13.88 ml, 0.1 mol) and Z(OMe)-Val-OH (14.60 g, 50 mmol). After addition of DCC (12.40 g, 60 mmol), the mixture was stirred for 48 hr, filtered and the filtrate was concentrated. The residue was purified as mentioned above to give a powder (11.90 g, 38%), m.p. 122-124°, $[\alpha]_D^{22} - 37.6^\circ$ (c 1.0 in DMF), Rf₁ 0.22 (Found: C, 59.44; H, 7.30; N, 13.49. C₃₁H₄₄N₆O₈ requires C, 59.22; H, 7.05; N, 13.37%).

Z(OMe)-Val-Pro-Val-His-NHNH₂. ——— Z(OMe)-Val-Pro-Val-His-OMe (19.11 g, 30 mmol) dissolved in MeOH (60 ml) was treated with 80% hydrazine hydrate (11.3 ml, 6 equiv.) overnight. The

solution was concentrated and the residue was treated with ether. Recrystallization from MeOH and ether afforded the hydrazide as a powder (18.10 g, 95%), m.p. 138-140°, $[\alpha]_D^{22} - 47.1^\circ$ (c 0.9 in DMSO), Rf_1 0.11 (Found: C, 56.12; H, 7.07; N, 17.34. $C_{30}H_{44}N_8O_{7.1/2}H_2O$ requires C, 56.49; H, 7.11; N, 17.57%).

Z(OMe)-Asn-Pro-Tyr-OMe. ——— Z-Pro-Tyr-OMe¹²⁷ (4.26 g, 10 mmol) in MeOH (30 ml) and 1N HCl (10 ml, 1 equiv.) was hydrogenated as usual. The solution was filtered, the filtrate was concentrated and the residue (Rf_1 0.29) was dissolved in DMF (30 ml) together with Et_3N (1.4 ml, 10 mmol) and Z(OMe)-Asn-ONP (4.17 g, 10 mmol). After stirring for 24 hr, the solution was concentrated and the residue was purified by procedure A followed by precipitation from AcOEt with ether to afford an amorphous powder (2.50 g, 44%). $[\alpha]_D^{20} - 41.9^\circ$ (c 0.9 in DMF), Rf_1 0.61 (Found: C, 57.83; H, 6.22; N, 10.01. $C_{28}H_{34}N_4O_{9.1/2}H_2O$ requires C, 58.02; H, 6.08; N, 9.66%). Use of excess Et_3N in the reaction afforded a heterogeneous oily product.

Z(OMe)-Asn-Pro-Tyr-NHNH₂. ——— Z(OMe)-Asn-Pro-Tyr-OMe (7.0 g, 12 mmol) in MeOH (30 ml) was treated with 80% hydrazine hydrate (4.5 ml, 6 equiv.) overnight. The gelatinous mass formed was recrystallized from MeOH to give the tripeptide hydrazide (3.71 g, 53%), m.p. 177-180°, $[\alpha]_D^{22} - 45.3^\circ$ (c 1.0 in DMF), Rf_1 0.33 (Found: C, 55.82; H, 6.41; N, 14.53. $C_{27}H_{34}N_6O_{8.1/2}H_2O$ requires C, 55.95; H, 6.09; N, 14.50%).

Section 2. Synthesis of the Protected Hexatriaconta-peptide Ester (Positions 89-124)

Z(OMe)-Val-Ala-OMe. ————— A mixed anhydride (prepared from 23.90 g, 85 mmol of Z(OMe)-Val-OH) in dry THF (200 ml) was added to an ice-chilled solution of H-Ala-OMe (prepared from 10.70 g, 85 mmol of the hydrochloride with 11.7 ml, 85 mmol of Et₃N) in DMF (80 ml) and the mixture was stirred in an ice-bath for 1 hr at room temperature for 4 hr. The solvent was evaporated and the residue was purified by procedure A followed by recrystallization from AcOEt and ether to give the protected dipeptide ester (19.91 g, 64%), m.p. 167-179°, $[\alpha]_D^{20}$ - 43.1° (c 0.8 in MeOH), Rf_1 0.85 (Found: C, 58.95; H, 7.31; N, 7.79. C₁₈H₂₆N₂O₆ requires C, 59.00; H, 7.15; N, 7.65%).

Z(OMe)-Val-Ala-NHNH₂ [4]. ————— Z(OMe)-Val-Ala-OMe (19.90 g, 54 mmol) in MeOH was treated with 80% hydrazine hydrate (27.0 ml, 8 equiv.) for 24 hr. Precipitation of the resulting solid from DMF with MeOH gave the dipeptide hydrazide (17.42 g, 88%), m.p. 240-243°, $[\alpha]_D^{22}$ - 6.6° (c 0.7 in DMSO), Rf_1 0.54 (Found: C, 55.58; H, 7.40; N, 15.28. C₁₇H₂₆N₄O₅ requires C, 55.72; H, 7.15; N, 15.29%).

Z(OMe)-Ile-Ile-OMe. ————— A mixed anhydride (prepared from 16.50 g, 56 mmol of Z(OMe)-Ile-OH) in dry THF (100 ml) was added to an ice-chilled solution of H-Ile-OMe (prepared from 10.20 g, 56 mmol of the hydrochloride with 7.7 ml, 56 mmol of Et₃N) in DMF (100 ml). After stirring in an ice-bath for 1 hr and at room temperature for 4 hr, the solution was concentrated and the residue was purified by procedure A followed by recrystallization from AcOEt and ether to give the protected dipeptide ester (16.31 g, 69%), m.p. 114-117°, $[\alpha]_D^{22}$ - 19.6° (c 1.0 in MeOH), Rf_1 0.98, Rf_2 0.87 (Found: C, 62.47; H, 8.23; N, 6.80. C₂₂H₃₄N₂O₆ requires C, 62.54;

H, 8.11; N, 6.63%).

Z(OMe)-Ile-Ile-NHNH₂ [5]. — Z(OMe)-Ile-Ile-OMe (16.30 g, 39 mmol) in MeOH (150 ml) was treated with 80 % hydrazine hydrate (19.5 ml, 8 equiv.) at 50° for 5 hr and then at room temperature overnight. The resulting solid was precipitated from DMF with MeOH to give the hydrazide (12.02 g, 73%), m.p. 234-237°, $[\alpha]_D^{22} = -4.5^\circ$ (c 1.1 in DMSO), R_f_1 0.56 (Found: C, 59.68; H, 8.22; N, 13.14. C₂₁H₃₄N₄O₅ requires C, 59.69; H, 8.11; N, 13.26%). No D-allo-Ile was detected in the 6N HCl (24 hr) hydrolysate.

Z(OMe)-Lys(Z)-His-OMe. — DCC (22.09 g, 0.11 mol) was added to a mixture of Z(OMe)-Lys(Z)-OH (44.45 g, 0.1 mol) and H-His-OMe (prepared from 20.52 g, 0.1 mol of the dihydrochloride with 27.6 ml, 0.2 mol of Et₃N) in DMF (350 ml) and the solution, after stirring for 48 hr, was filtered; the filtrate was concentrated and the residue was treated with ether and 5% NaHCO₃ to afford a powder, which was washed with 5% NaHCO₃, H₂O and ether. The product was further treated with MeOH containing 3% AcOH at 50° for 7 hr and recrystallized from MeOH and AcOEt to give the dipeptide ester (44.55 g, 75%), m.p. 140-142°, $[\alpha]_D^{22} = 7.8^\circ$ (c 0.9 in DMF), R_f_1 0.60 (Found: C, 60.74; H, 6.18; N, 11.47. C₃₀H₃₇N₅O₈ requires C, 60.49; H, 6.26; N, 11.76%).

Z(OMe)-Asn-Lys(Z)-His-OMe. — Z(OMe)-Lys(Z)-His-OMe (29.78 g, 50 mmol) was treated with TFA-anisole (52 ml-14 ml) as usual and dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets in vacuo for 3 hr and dissolved in DMF (200 ml) together with Et₃N (13.8 ml, 0.1 mol) and Z(OMe)-Asn-ONP (25.0 g, 60 mmol). After 48 hr, evaporation of the solvent followed by trituration of the residue with AcOEt afforded a powder, which was washed with 5% Na₂CO₃ and H₂O and then recryst-

crystallized from MeOH and AcOEt to give the protected tripeptide ester (25.47 g, 72%), m.p. 184-187°, $[\alpha]_D^{22} + 4.6^\circ$ (c 0.9 in DMF), Rf_1 0.43 (Found: C, 56.39; H, 6.21; N, 13.53. $C_{34}H_{43}N_7O_{10} \cdot H_2O$ requires C, 56.11; H, 6.23; N, 13.47%).

Z(OMe)-Asn-Lys(Z)-His-NHNH₂, Z(OMe)-(RNase 103-105)-NHNH₂ [6].

———— Z(OMe)-Asn-Lys(Z)-His-OMe (14.90 g, 21 mmol) dissolved in MeOH (150 ml) was treated with 80% hydrazine hydrate (8.4 ml, 6.4 equiv.) overnight. A gelatinous mass was formed and was recrystallized from MeOH to give the tripeptide hydrazide (12.40 g, 83%), m.p. 180-181°, $[\alpha]_D^{23} - 6.9^\circ$ (c 0.6 in DMSO), Rf_1 0.48. Amino acid analysis: Asp 1.10, Lys 1.00, His 0.86 (average recovery 90%), (Found: C, 54.71; H, 6.22; N, 17.20. $C_{33}H_{43}N_9O_9 \cdot H_2O$ requires C, 54.46; H, 6.23; N, 17.32%).

Z-Thr-Gln-Ala-OMe. ————— In the presence of 1N HCl (40 ml, 1 equiv.), Z-Gln-Ala-OMe⁷⁴ (16.02 g, 40 mmol) in THF (200 ml) was hydrogenated over a Pd catalyst in the usual manner for 8 hr. The filtered solution was neutralized with Et₃N (5.5 ml, 40 mmol). After addition of Z-Thr-OPCP (21.96 g, 44 mmol) and Et₃N (5.5 ml, 40 mmol), the mixture was stirred for 48 hr and the solvent was evaporated. Treatment of the residue with ether afforded a fine powder, which was purified by procedure B followed by recrystallization from MeOH to give the protected tripeptide ester (10.80 g, 58%), m.p. 199-202°, $[\alpha]_D^{22} - 9.0^\circ$ (c 1.0 in DMF), Rf_1 0.32, Rf_4 0.58 (Found: C, 54.15; H, 6.64; N, 12.03. $C_{21}H_{30}N_4O_8$ requires C, 54.07; H, 6.48; N, 12.01%).

Z-Thr-Thr-Gln-Ala-OMe. ————— In the presence of 1N HCl (44 ml, 1 equiv.), Z-Thr-Gln-Ala-OMe (20.53 g, 44 mmol) in THF (150 ml) was hydrogenated over a Pd catalyst as mentioned above. To this filtered solution were added Et₃N (12.1 ml, 88 mmol) and Z-Thr-OPCP

(23.95 g, 48 mmol) in DMF (120 ml) and the mixture was stirred for 48 hr. Evaporation of the solvent followed by trituration of the residue with AcOEt afforded a gelatinous mass, which was purified by procedure B. Recrystallization from MeOH afforded the protected tetrapeptide ester (18.92 g, 76%), m.p. 238-239°, $[\alpha]_D^{22} + 4.9^\circ$ (c 1.0 in DMF), Rf_1 0.46 (Found: C, 52.76; H, 6.76; N, 12.42. $C_{25}H_{37}N_5O_{10}$ requires C, 52.25; H, 6.58; N, 11.72%).

Z(OMe)-Lys(Z)-Thr-Thr-Gln-Ala-OMe. — Z-Thr-Thr-Gln-Ala-OMe (8.82 g, 16 mmol) in MeOH (80 ml) containing 1N HCl (16 ml, 1 equiv.) was hydrogenated as mentioned above. The filtered solution was concentrated and the residue was dissolved in DMF (100 ml) together with Et_3N (4.3 ml, 31 mmol) and Z(OMe)-Lys(Z)-OTCP (10.70 g, 17 mmol). After stirring for 48 hr, the solution was concentrated. Trituration of the residue with AcOEt afforded a gelatinous solid, which was purified by procedure B followed by precipitation from DMF with AcOEt to give the protected pentapeptide ester (8.87 g, 67%), m.p. 169-172°, $[\alpha]_D^{22} + 3.9^\circ$ (c 1.2 in DMF), Rf_1 0.39 (Found: C, 54.67; H, 6.98; N, 11.29. $C_{40}H_{57}N_7O_{14} \cdot H_2O$ requires C, 54.72; H, 6.77; N, 11.17%).

Z(OMe)-Lys(Z)-Thr-Thr-Gln-Ala-NHNH₂, Z(OMe)-(RNase 98-102)-NHNH₂ [7]. — Z(OMe)-Lys(Z)-Thr-Thr-Gln-Ala-OMe (8.60 g, 10 mmol) in DMF (60 ml) was treated with 80% hydrazine hydrate (6.2 ml, 10 equiv.) overnight. The resulting gelatinous mass was collected by filtration and washed with MeOH to give the hydrazide (7.78 g, 90%), m.p. 206-209°, $[\alpha]_D^{22} + 0.8^\circ$ (c 0.9 in DMSO), Rf_1 0.16. Amino acid analysis: Lys 1.14, Thr 2.12, Glu 1.10, Ala 1.00 (average recovery 83%). (Found: C, 53.45; H, 6.91; N, 14.48. $C_{39}H_{57}N_9O_{13} \cdot H_2O$ requires C, 53.35; H, 6.77; N, 14.36%).

Z(OMe)-Cys(MBzl)-Ala-Tyr-OMe. — Z-Ala-Tyr-OMe⁸⁰ (20.02 g,

mmol) in MeOH (150 ml) and 1N HCl (50 ml, 1 equiv.) was hydrogenated over a Pd catalyst for 8 hr. The catalyst was removed by filtration, the filtrate was concentrated and the residue was dissolved in DMF (150 ml). Et₃N (6.9 ml, 50 mmol), Z(OMe)-Cys(MBzl)- (20.27 g, 50 mmol) and DCC (11.30 g, 55 mmol) were successively added and the mixture was stirred for 48 hr. The solution was filtered, the filtrate was concentrated and the resulting solid residue was purified by procedure B followed by recrystallization from oxane and MeOH to give the protected tripeptide ester (21.05 g, %), m.p. 182-185°, $[\alpha]_D^{23}$ - 8.5° (c 1.1 in DMF), Rf₁ 0.73 (Found: 60.42; H, 6.02; N, 6.53. C₃₃H₃₉N₃O₉S requires C, 60.63; H, 6.01; 6.43%).

Z(OMe)-Asn-Cys(MBzl)-Ala-Tyr-OMe. ——— Z(OMe)-Cys(MBzl)-a-Tyr-OMe (16.22 g, 25 mmol) was treated with TFA-anisole (20 ml-ml) as usual and dry ether was added. The resulting oily precipitate was dried over KOH pellets in vacuo for 3 hr and dissolved in F (160 ml) together with Et₃N (6.9 ml, 50 mmol) and Z(OMe)-Asn-P (10.34 g, 25 mmol). After stirring for 48 hr, the solution was concentrated and the solid residue was purified by procedure B followed by precipitation from DMF with AcOEt to give the protected tripeptide ester (16.33 g, 86%), m.p. 210-213°. $[\alpha]_D^{22}$ - 16.3° (c 1.0 in DMF), Rf₁ 0.54 (Found: C, 57.42; H, 6.00; N, 9.27. C₃₇H₄₅O₁₁S requires C, 57.87; H, 5.90; N, 9.12%).

Z(OMe)-Pro-Asn-Cys(MBzl)-Ala-Tyr-OMe. ——— Z(OMe)-Asn-Cys(MBzl)-Ala-Tyr-OMe (11.63 g, 15 mmol) was treated with TFA-anisole (5 ml- 6 ml) as usual and dry ether was added. The resulting powder was dried over KOH pellets in vacuo for 3 hr and dissolved in F (100 ml) together with Et₃N (4.1 ml, 30 mmol) and Z(OMe)-Pro-P (6.67 g, 17 mmol). After stirring for 48 hr, the solution was concentrated and the residue was treated with ether. The resulting

powder was purified by procedure B followed by precipitation from DMF with AcOEt to give the protected pentapeptide ester (11.48 g, 89%), m.p. 156–159°, $[\alpha]_D^{23}$ -33.4° (c 0.8 in DMF), Rf_1 0.46 (Found: C, 57.16; H, 6.07; N, 9.58. $C_{42}H_{52}N_6O_{12}S \cdot H_2O$ requires C, 57.13; H, 6.16; N, 9.95%).

Z(OMe)-Tyr-Pro-Asn-Cys(MBzl)-Ala-Tyr-OMe. ——— The above protected pentapeptide ester (16.66 g, 19 mmol) was treated with TFA-anisole (50 ml–18 ml) as usual and the N^α -deprotected peptide, isolated as stated above, was dissolved in DMF (120 ml) containing Et_3N (5.2 ml, 38 mmol). To this ice-chilled solution, the azide (prepared from 8.34 g, 23 mmol of Z(OMe)-Tyr-NHNH₂) in DMF (50 ml) was added. After stirring for 24 hr, the solution was concentrated and the residue was triturated with 5% citric acid and ether. The resulting powder was purified by procedure B followed by recrystallization from MeOH to give the hexapeptide ester (17.88 g, 89%), m.p. 139–144°, $[\alpha]_D^{23}$ - 33.9° (c 1.0 in DMF), Rf_1 0.61 (Found: C, 58.41; H, 5.90; N, 9.39. $C_{51}H_{61}N_7O_{14}S \cdot H_2O$ requires C, 58.54; H, 6.07; N, 9.39%).

Z(OMe)-Tyr-Pro-Asn-Cys(MBzl)-Ala-Tyr-NHNH₂, Z(OMe)-(RNase 92-97)-NHNH₂ [8]. ——— The above protected hexapeptide ester (15.31 g, 15 mmol) dissolved in DMF-MeOH (50 ml– 100 ml) was treated with 80% hydrazine hydrate (7.3 ml, 8 equiv.) overnight. Precipitation of the resulting mass from DMF with MeOH afforded the hydrazide (13.29 g, 87%), m.p. 174–177°, $[\alpha]_D^{21}$ - 38.8° (c 0.8 in DMSO), Rf_1 0.72. Amino acid analysis: Tyr 1.96, Pro 1.00, Asp 1.18, Ala 1.20, average recovery 82%, (Found: C, 57.19; H, 5.83; N, 12.27. $C_{50}H_{61}N_9O_{13} \cdot H_2O$ requires C, 57.40; H, 6.07; N, 12.05%).

Z(OMe)-Ser-Lys(Z)-OMe. ——— The azide (prepared from 16.20 g, 57 mmol of Z(OMe)-Ser-NHNH₂) in DMF (80 ml) and Et_3N (7.9 ml,

57 mmol) were added to a solution of H-Lys(Z)-OMe (prepared from 18.90 g, 57 mmol of the hydrochloride with 7.9 ml, 57 mmol of Et₃N) in DMF (150 ml). After stirring for 24 hr, the solution was concentrated. The residue was purified by procedure A followed by recrystallization from AcOEt and ether to give the protected dipeptide ester (20.61 g, 66%), m.p. 104-106°, $[\alpha]_D^{23} - 6.9^\circ$ (c 1.0 in DMF), Rf_1 0.88 (Found: C, 59.68; H, 6.43; N, 7.71. C₂₇H₃₅N₃O₉ requires C, 59.44; H, 6.47; N, 7.70%).

Z(OMe)-Ser-Ser-Lys(Z)-OMe. ——— Z(OMe)-Ser-Lys(Z)-OMe (20.41 g, 37 mmol) was treated with TFA-anisole (80 ml-20 ml) as usual and dry ether was added. The resulting powder was dissolved in DMF (150 ml) containing Et₃N (5.1 ml, 37 mmol). To this ice-chilled solution, the azide (prepared from 11.64 g, 41 mmol of Z(OMe)-Ser-NHNH₂) in DMF (50 ml) and Et₃N (5.1 ml, 37 mmol) were added. After stirring for 24 hr, the solution was concentrated and the residue was purified by procedure A followed by recrystallization from MeOH and ether to give the protected tripeptide ester (21.40 g, 91%), m.p. 94-96°, $[\alpha]_D^{22} = 7.0^\circ$ (c 0.9 in DMF), Rf_1 0.67 (Found: C, 56.65; H, 6.45; N, 9.11. C₃₀H₄₀N₄O₁₁ requires C, 56.95; H, 6.37; N, 8.86%).

Z(OMe)-Ser-Ser-Lys(Z)-NHNH₂, Z(OMe)-(RNase 89-91)-NHNH₂ [9].
 ——— Z(OMe)-Ser-Ser-Lys(Z)-OMe (21.10 g, 33 mmol) in MeOH (200 ml) was treated with 80% hydrazine hydrate (16 ml, 8 equiv.) overnight. Precipitation of the resulting mass from DMF with MeOH gave the hydrazide (19.03 g, 87%), m.p. 204-206°, $[\alpha]_D^{22} + 4.7^\circ$ (c 0.8 in DMSO), Rf_1 0.39. Amino acid analysis: Ser 1.62, Lys 1.00 average recovery 90% (Found: C, 54.76; H, 6.21; N, 13.48. C₂₉H₄₀N₆O₁₀ requires C, 55.05; H, 6.37; N, 13.28%).

Z(OMe)-Val-Ala-Cys(MBzl)-Glu(OBzl)-Gly-Asn-Pro-Tyr-Val-Pro-

Val-His-Phe-Asp(OBzl)-Ala-Ser-Val-OBzl, Z(OMe)-(RNase 108-124)-OBzl. ——— Z(OMe)-(RNase 110-124)-OBzl (12.90 g, 5.9 mmol) was treated with TFA-anisole (40 ml-10 ml) as usual and dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets in vacuo for 3 hr and then dissolved in DMF (100 ml) containing Et₃N (1.63 ml, 11.8 mmol). To this ice-chilled solution, the azide (prepared from 2.59 g, 7.1 mmol of Z(OMe)-Val-Ala-NHNH₂) in DMF (10 ml) and Et₃N (0.98 ml, 7.1 mmol) were added. After stirring for 48 hr, the solution was concentrated. Trituration of the residue with AcOEt and H₂O afforded a powder, which was purified by procedure B followed by precipitation twice from DMF with MeOH to give the protected heptadecapeptide ester (10.10 g, 72%), m.p. 238-243°, $[\alpha]_D^{22}$ - 50.9° (c 0.8 in DMSO), Rf₁ 0.46 (Found: C, 59.75; H, 6.73; N, 11.64; S, 1.50. C₁₁₉H₁₅₂N₂₀O₂₉S. 2H₂O requires C, 59.68; H, 6.57; N, 11.70; S, 1.34%).

Z(OMe)-Ile-Ile-Val-Ala-Cys(MBzl)-Glu(OBzl)-Gly-Asn-Pro-Tyr-Val-Pro-Val-His-Phe-Asp(OBzl)-Ala-Ser-Val-OBzl, Z(OMe)-(RNase 106-124)-OBzl. ——— Z(OMe)-(RNase 108-124)-OBzl (12.01 g, 5 mmol) was treated with TFA-anisole (40 ml-12 ml) and the N^α-deprotected peptide, isolated as stated above, was then dissolved in DMF-HMPA (100 ml, 3:2 v/v) containing Et₃N (1.38 ml, 10 mmol). To this ice-chilled solution, the azide (prepared from 5.08 g, 12 mmol of Z(OMe)-Ile-Ile-NHNH₂) in DMF (20 ml) and Et₃N (1.66 ml, 12 mmol) were added. After stirring for 48 hr, the solution was concentrated and the residue was treated with 5% NaHCO₃ and ether to afford a fine powder, which was purified by procedure B followed by precipitation twice from DMSO with MeOH to give the protected nonadecapeptide ester (10.37 g, 79%), m.p. 249-259°, $[\alpha]_D^{22}$ - 36.8° (c 0.8 in DMSO), Rf₁ 0.50 (Found: C, 60.03; H, 6.78; N, 11.79. C₁₃₁H₁₇₄N₂₂O₃₁S. 2H₂O requires C, 60.01; H, 6.85; N, 11.76%).

Z(OMe)-Asn-Lys(Z)-His-Ile-Ile-Val-Ala-Cys(MBzl)-Glu(OBzl)-Gly-Asn-Pro-Tyr-Val-Pro-Val-His-Phe-Asp(OBzl)-Ala-Ser-Val-OBzl, Z(OMe)-(RNase 103-124)-OBzl.—— Z(OMe)-(RNase 106-124)-OBzl (12.03 g, 4.6 mmol) was treated with TFA-anisole (48 ml-12 ml) and the N^α-deprotected peptide, isolated as mentioned above, was dissolved in DMF-HMPA (30 ml, 3:1 v/v) containing Et₃N (1.3 ml, 9.2 mmol). To this ice-chilled solution, the azide (prepared from 8.02 g, 11 mmol of Z(OMe)-Asn-Lys(Z)-His-NHNH₂) in DMF (80 ml) and N-methylmorpholine (1.21 ml, 11 mmol) were added. After stirring for 48 hr, the solution was concentrated and the residue was treated with 5% NaHCO₃ to give a fine powder, which was purified by procedure B followed by precipitation twice from DMSO with MeOH to give the protected docosapeptide ester (11.72 g, 81%), m.p. 251-256°, $[\alpha]_D^{23}$ - 35.5° (c 0.9 in DMSO), Rf₁ 0.61, Rf₃ 0.85 (Found: C, 59.00; H, 6.94; N, 13.15; S, 1.28. C₁₅₅H₂₀₅N₂₉O₃₇S.3H₂O requires C, 59.05; H, 6.75; N, 12.89; S, 1.02%).

Z(OMe)-Lys(Z)-Thr-Thr-Gln-Ala-Asn-Lys(Z)-His-Ile-Ile-Val-Ala-Cys(MBzl)-Glu(OBzl)-Gly-Asn-Pro-Tyr-Val-Pro-Val-His-Phe-Asp(OBzl)-Ala-Ser-Val-OBzl, Z(OMe)-(RNase 98-124)-OBzl.—— Z(OMe)-(RNase 103-124)-OBzl (13.00 g, 4.1 mmol) was treated with TFA-anisole (50 ml-13 ml) and the N^α-deprotected peptide, isolated as mentioned above, was dissolved in DMF-DMSO (100 ml, 1:1 v/v) containing Et₃N (1.71 ml, 12.4 mmol). To this ice-chilled solution, the azide (prepared from 9.04 g, 10.3 mmol of Z(OMe)-Lys(Z)-Thr-Thr-Gln-Ala-NHNH₂) in DMF (60 ml) and Et₃N (1.42 ml, 10.3 mmol) were added. After stirring for 48 hr, the solution was concentrated and the residue was treated with 5% NaHCO₃ and ether to give a fine powder, which was purified by procedure B followed by precipitation twice from DMSO with MeOH to give the protected heptacosapeptide ester (14.09 g, 89%), m.p. 250-256°. $[\alpha]_D^{23}$ - 35.9° (c 1.0 in DMSO), Rf₁ 0.54, Rf₃ 0.78 (Found: C, 57.69; H, 6.64; N, 12.85; S, 1.14. C₁₈₅H₂₅₀N₃₆O₄₇S.

4H₂O requires C, 57.95; H, 6.78; N, 13.15; S, 0.84%).

Z(OMe)-Tyr-Pro-Asn-Cys(MBzl)-Ala-Tyr-Lys(Z)-Thr-Thr-Gln-Ala-Asn-Lys(Z)-His-Ile-Ile-Val-Ala-Cys(MBzl)-Glu(OBzl)-Gly-Asn-Pro-Tyr-Val-Pro-Val-His-Phe-Asp(OBzl)-Ala-Ser-Val-OBzl, Z(OMe)-(RNase 92-124)-OBzl. ——— Z(OMe)-(RNase 98-124)-OBzl (13.88 g, 3.6 mmol) was treated with TFA-anisole (50 ml-13 ml) and the N^α-deprotected peptide, isolated as mentioned above, was dissolved in DMF-DMSO (90 ml, 1:1 v/v) containing Et₃N (1.50 ml, 10.9 mmol). To this ice-chilled solution, the azide (prepared from 9.30 g, 9 mmol of Z(OMe)-Tyr-Pro-Asn-Cys(MBzl)-Ala-Tyr-NHNH₂) in DMF (50 ml) and Et₃N (1.25 ml, 9 mmol) were added. After stirring for 48 hr, the solution was concentrated and the residue was treated with ether and 5% NaHCO₃. The resulting powder was purified by procedure B followed by precipitation (three times) from DMSO with MeOH to give the protected tritriacontapeptide ester (14.89 g, 87%), m.p. 236-241°, [α]_D²³ -40.0° (c 0.8 in DMSO), R_f 0.71 (Found: C, 57.24; H, 6.47; N, 12.81. C₂₂₆H₂₉₉N₄₃O₅₇S₂.8H₂O requires C, 57.28; H, 6.66; N, 12.71%).

Z(OMe)-Ser-Ser-Lys(Z)-Tyr-Pro-Asn-Cys(MBzl)-Ala-Tyr-Lys(Z)-Thr-Thr-Gln-Ala-Asn-Lys(Z)-His-Ile-Ile-Val-Ala-Cys(MBzl)-Glu(OBzl)-Gly-Asn-Pro-Tyr-Val-Pro-Val-His-Phe-Asp(OBzl)-Ala-Ser-Val-OBzl, Z(OMe)-(RNase 89-124)-OBzl. ——— Z(OMe)-(RNase 92-124)-OBzl (12.20 g, 2.6 mmol) was treated with TFA-anisole (50 ml-12 ml) and the N^α-deprotected peptide, isolated as stated above, was dissolved in DMF-DMSO (100 ml, 1:1 v/v) containing Et₃N (1.06 ml, 7.7 mmol). To this ice-chilled solution, the azide (prepared from 4.08 g, 6.4 mmol of Z(OMe)-Ser-Ser-Lys(Z)-NHNH₂) in DMF (30 ml) and Et₃N (0.89 ml, 6.4 mmol) were added. After stirring for 48 hr, the solution was concentrated. The residue was purified as mentioned above and finally precipitated three times from DMSO with AcOEt to give the protected hexatriacontapeptide ester (12.33 g, 93%), m.p. 241-

250°, $[\alpha]_D^{23} - 36.1^\circ$ (c 0.8 in DMSO), Rf_3 0.76 (Found: C, 57.26; H, 5.52; N, 12.51; S, 1.44. $C_{246}H_{327}N_{47}O_{64}S_2 \cdot 7H_2O$ requires C, 57.29; H, 6.67; N, 12.77; S, 1.24%).

Trial experiments

Z-Ile-Val-Ala-OMe. ————— Z-Val-Ala-OMe¹²⁸ (4.70 g, 14 mmol) was treated with 2.7 N HBr-AcOH (12 ml) at room temperature for 40 min. The HBr salt precipitated by ether was dissolved in DMF (25 ml) together with Et_3N (1.9 ml, 14 mmol) and Z-Ile-OH (3.71 g, 14 mmol). After addition of DCC (3.07 g, 15 mmol), the mixture was stirred for 24 hr, filtered and the filtrate was concentrated. Treatment of the residue with ether afforded a gelatinous mass, which was purified by procedure B followed by recrystallization from MeOH to give the protected tripeptide ester (4.72 g, 75%), n.p. 190-194°, $[\alpha]_D^{23} - 8.7^\circ$ (c 0.6 in DMF), Rf_1 0.77 (Found: C, 51.90; H, 7.96; N, 9.63. $C_{23}H_{35}N_3O_6$ requires C, 61.45; H, 7.85; N, 9.35%).

Z(OMe)-Ile-Ile-Val-Ala-OMe. ————— Z-Ile-Val-Ala-OMe (1.53 g, 3.4 mmol) in THF (30 ml) containing 1N HCl-dioxane (3.4 ml) was hydrogenated over a Pd catalyst for 8 hr and the catalyst was removed by filtration. To this filtrate were added Et_3N (0.47 ml, 3.4 mmol), Z(OMe)-Ile-OH (1.01 g, 3.4 mmol) and DCC (0.77 g, 3.7 mmol) and the mixture was stirred overnight. After filtration, the filtrate was concentrated. Trituration of the residue with AcOEt afforded a powder, which was purified by procedure B followed by precipitation from DMF with AcOEt to give the protected tetrapeptide ester (1.34 g, 66%), m.p. 241-246°, $[\alpha]_D^{22} - 25.0^\circ$ (c 1.1 in DMSO), Rf_1 0.52, Rf_3 0.61 (Found: C, 60.52; H, 8.41; N, 9.49. $C_{30}H_{48}N_4O_8$ requires C, 60.79; H, 8.16; N, 9.45%).

Z(OMe)-Ile-Ile-Val-Ala-NHNH₂ (positions 106-109). —————

Z(OMe)-Ile-Ile-Val-Ala-OMe (0.84 g, 1.4 mmol) in DMF (8 ml) was treated with 80% hydrazine hydrate (0.8 ml, 9 equiv.) overnight. A gelatinous mass formed and was washed with MeOH to give the hydrazide, which was less soluble even in hot DMF (0.77 g, 92%), m.p. 277° dec., $[\alpha]_D^{22}$ - 29.3° (c 0.6 in DMSO), Rf_1 0.44 (Found: C, 59.01; H, 8.35; N, 14.30. $C_{29}H_{48}N_6O_7$ requires C, 58.76; H, 8.16; N, 14.18 %).

Z(OMe)-Pro-Asn-Cys(MBzl)-Ala-Tyr-NHNH₂ (positions 93-97) ———
 — Z(OMe)-Pro-Asn-Cys(MBzl)-Ala-Tyr-OMe (8.83 g, 10 mmol) in DMF (50 ml) was treated with 80% hydrazine hydrate (6 ml, 10 equiv.) overnight. A gelatinous mass, formed by addition of MeOH, was collected by filtration and washed with MeOH to give the hydrazide (7.62 g, 86%), m.p. 208-211°, $[\alpha]_D^{24}$ - 38.5° (c 0.9 in DMSO), Rf_1 0.44, Rf_3 0.89 (Found: C, 56.08; H, 6.33; N, 12.68. $C_{41}H_{52}N_8O_{11}S \cdot H_2O$ requires C, 55.76; H, 6.16; N, 12.69%).

Z(OMe)-Lys(Z)-Tyr-OMe. ——— DCC (4.08 g, 20 mmol) was added to a mixture of Z(OMe)-Lys(Z)-OH (8.88 g, 20 mmol) and H-Tyr-OMe (prepared from 4.64 g, 20 mmol of the hydrochloride with 2.8 ml, 20 mmol of Et₃N) in DMF (80 ml). After 24hr, the solution was filtered, the filtrate was concentrated and the residue was purified by procedure A. Recrystallization from AcOEt and ether afforded the dipeptide ester (4.39 g, 55%), m.p. 106-109°, $[\alpha]_D^{22}$ - 7.3° (c 1.0 in MeOH), Rf_1 0.62 (Found: C, 64.06; H, 6.57; N, 6.90. $C_{33}H_{39}N_3O_9$ requires C, 63.75; H, 6.32; N, 6.76%).

Z(OMe)-Ser-Lys(Z)-Tyr-OMe. ——— Z(OMe)-Lys(Z)-Tyr-OMe (2.46 g, 6.2 mmol) was treated with TFA-anisole (5 ml-2 ml) as usual and dry ether was added. An oily precipitate was dried over KOH pellets in vacuo for 3 hr and dissolved in DMF (40 ml) together with Et₃N (1.7 ml, 12 mmol) and Z(OMe)-Ser-OPCP (3.84 g, 7.4 mmol). After

stirring for 24 hr, the solution was concentrated and the residue was purified by procedure A. Recrystallization from a mixture of dioxane and MeOH gave the protected tripeptide ester (2.99 g, 68%), m.p. 178-181°, $[\alpha]_D^{22} - 2.3^\circ$ (c 0.9 in DMF), Rf_1 0.76 (Found: C, 60.72; H, 6.43; N, 7.92. $C_{36}H_{44}N_4O_{11}$ requires C, 61.00; H, 6.26; N, 7.91%).

Z(OMe)-Ser-Ser-Lys(Z)-Tyr-OMe. ——— Z(OMe)-Ser-Lys(Z)-Tyr-OMe (2.13 g, 3 mmol) was treated with TFA-anisole (3 ml- 1.5 ml) as usual and dry ether was added. The resulting powder was dissolved in DMF (20 ml) together with Et_3N (0.8 ml, 6 mmol) and Z(OMe)-Ser-OPCP (1.56 g, 3 mmol). After stirring for 24 hr, the solution was concentrated and the residue was treated with AcOEt. The resulting powder was purified by procedure B and recrystallized from MeOH and AcOEt to afford the protected tetrapeptide ester (1.88 g, 79%), m.p. 134-137°, $[\alpha]_D^{22} + 6.9^\circ$ (c 0.9 in DMF), Rf_1 0.55 (Found: C, 59.10; H, 6.29; N, 8.72. $C_{39}H_{49}N_5O_{13}$ requires C, 58.86; H, 6.20; N, 8.80%).

Z(OMe)-Ser-Ser-Lys(Z)-Tyr-NHNH₂ (position 89-92). ——— Z(OMe)-Ser-Ser-Lys(Z)-Tyr-OMe (1.26 g, 1.6 mmol) in MeOH (12 ml) was treated with 80% hydrazine hydrate (1.0 ml, 10 equiv.) overnight. A gelatinous mass formed and was collected by filtration and washed with MeOH to give the hydrazide (1.10 g, 87%), m.p. 206-208°, $[\alpha]_D^{22} + 2.7^\circ$ (c 0.7 in DMSO), Rf_1 0.62 (Found: C, 56.62; H, 6.42; N, 11.41. $C_{38}H_{49}N_7O_{12} \cdot H_2O$ requires C, 56.07; H, 6.32; N, 12.04%).

Z(OMe)-Pro-Asn-Cys(MBzl)-Ala-Tyr-(98-124)-OBzl, Z(OMe)-(RNase 93-124)-OBzl. ——— Z(OMe)-(RNase 98-124)-OBzl (4.20 g, 1.1 mmol) was treated with TFA-anisole (15 ml-4 ml) and the N^α -deprotected peptide, isolated as mentioned above, was dissolved in DMF-DMSO (30 ml, 1:1, v/v) containing Et_3N (0.46 ml, 3.3 mmol). To this

ice-chilled solution, the azide (prepared from 2.37 g, 2.7 mmol of Z(OMe)-Pro-Asn-Cys(MBzl)-Ala-Tyr-NHNH₂ in DMF (10 ml) and Et₃N (0.37 ml, 2.7 mmol) were added. After stirring for 48 hr, the solution was concentrated and the residue was treated with ether and 5% NaHCO₃. The resulting powder was purified by procedure B followed by precipitation twice from DMSO with MeOH to give the protected dotriacontapeptide ester (3.86 g, 78%), m.p. 241-251°, [α]_D²² -53.6° (c 0.9 in DMSO), Rf₁ 0.61, Rf₃ 0.79. Amino acid analysis: Asp 4.03, Thr 1.66, Ser 0.94, Glu 2.06, Pro 3.04, Gly 1.00, Ala 3.92, Val 4.24, Ile 1.63, Tyr 1.87, Phe 1.00, Lys 1.70, His 1.97, average recovery 86%, (Found: C, 57.34; H, 6.86; N, 13.07; S, 1.66. C₂₁₇H₃₀₂N₄₂O₅₅S₂·6H₂O requires C, 57.42; H, 6.71; N, 12.96; S, 1.41%).

Alternative Synthesis of Z(OMe)-(RNase 89-124)-OBzl.

The above protected dotriacontapeptide ester, Z(OMe)-(RNase 93-124)-OBzl, (3.86 g, 0.85 mmol) was treated with TFA-anisole (16 ml-4 ml) and the N^α-deprotected peptide, isolated as mentioned above, was dissolved in DMF-DMSO (20 ml, 1:1 v/v) containing Et₃N (0.35 ml, 2.6 mmol). To this ice-chilled solution, the azide (prepared from 1.69 g, 2.13 mmol of Z(OMe)-Ser-Ser-Lys(Z)-Tyr-NHNH₂) in DMF (15 ml) and Et₃N (0.29 ml, 2.13 mmol) were added. After 48 hr, an additional azide (3 equiv.) was added and the mixture was stirred for 48 hr. The solvent was evaporated and the product was isolated in essentially the same manner as mentioned above; yield 2.51 g (57%), m.p. 246°-251°, [α]_D²³ - 29.6° (c 0.7 in DMSO), Rf₁ 0.46, Rf₃ 0.76. Amino acid analysis: Asp 4.24, Thr 1.67, Ser 2.95, Glu 2.10 Pro 3.18, Gly 1.00, Ala 4.30, Val 4.08, Ile 1.70, Tyr 2.96, Phe 1.00, Lys 2.74, His 2.04 average recovery 91%.

Section 3. Synthesis of the Protected Hexapentacontapeptide Ester (Positions 69-124)

Z(OMe)-Thr-Gly-OH. ——— Z(OMe)-Thr-OPCP (53.16 g, 0.1 mol) dissolved in THF (250 ml) was added to a stirred solution of Gly (22.52 g, 0.3 mol) in H₂O (150 ml) containing Et₃N (48 ml, 0.35 mol). After 24 hr, the solvent was removed by evaporation and the product was isolated by procedure C. Recrystallization from AcOEt and ether gave the protected dipeptide (14.91 g, 44%), m.p. 113-115°, $[\alpha]_D^{23} + 7.6^\circ$ (c 0.8 in DMF), Rf_1 0.29 (Found: C, 52.67; H, 5.88; N, 8.11. C₁₅H₂₀N₂O₇ requires C, 52.93; H, 5.92; N, 8.23%).

Z(OMe)-Glu(OBzl)-Thr-Gly-OH. ——— Z(OMe)-Thr-Gly-OH (28.80 g, 85 mmol) dissolved in MeOH (100 ml) containing a few drops of AcOH was hydrogenated over a Pd catalyst for 8 hr. The filtered solution was concentrated and the residue was recrystallized from H₂O and MeOH to give H-Thr-Gly-OH (14.02 g, 94%). This dipeptide (6.34 g, 36 mmol) was dissolved in H₂O (50 ml) containing Et₃N (9.9 ml, 72 mmol) and a solution of Z(OMe)-Glu(OBzl)-ONP (18.81 g, 36 mmol) in THF (150 ml) was added. After stirring for 48 hr, the mixture was concentrated and the residue was purified by washing with 5% citric acid and H₂O followed by recrystallization from MeOH and AcOEt to yield the protected tripeptide (17.91 g, 89%), m.p. 122-124°, $[\alpha]_D^{22} + 1.5^\circ$ (c 0.7 in DMF), Rf_1 0.25 (Found: C, 57.89; H, 5.88; N, 7.65. C₂₇H₃₃N₃O₁₀ requires C, 57.95; H, 5.94; N, 7.51%).

Z(OMe)-Glu(OBzl)-Thr-Gly-NHNH-Troc. ——— DCC (2.47 g, 12 mmol) was added to a stirred mixture of Z(OMe)-Glu(OBzl)-Thr-Gly-OH (5.60 g, 10 mmol), Troc-NHNH₂ (2.49 g, 12 mmol) and HOBT (1.62 g, 12 mmol) in DMF (50 ml). After 48 hr, the solution was filtered, the filtrate was concentrated and the residue was purified by procedure A. Trituration with ether followed by recrystallization from

AcOEt and ether gave the substituted hydrazide (7.30 g, 97%), m.p. 87-89°, $[\alpha]_D^{23} -2.4^\circ$ (c 0.4 in DMF), Rf_1 0.62 (Found: C, 48.17; H, 4.98; N, 9.31, $C_{30}H_{36}N_5O_{11}Cl_3$ requires C, 48.10; H, 4.84; N, 9.35%).

Z(OMe)-Arg(MBS)-Glu(OBzl)-Thr-Gly-NHNH-Troc. —————

Z(OMe)-Glu(OBzl)-Thr-Gly-NHNH-Troc (10.74 g, 14 mmol) was treated with TFA-anisole (21 ml-5.2 ml) as usual, then dry ether was added. The resulting powder was dissolved in 4.2N HCl-dioxane (3.3 ml, 14 mmol), the solvent was evaporated and the residue was again treated with ether to afford HCl.H-Glu(OBzl)-Thr-Gly-NHNH-Troc as a powder, which was dissolved in DMF (40 ml) containing Et_3N (3.9 ml, 28 mmol). DCC (3.17 g, 15 mmol) was added to a stirred solution of Z(OMe)-Arg(MBS)-OH (7.83 g, 15 mmol) and DNP-OH (2.84 g, 15 mmol) in AcOEt (20 ml). After 5 hr, the solution was filtered, the filtrate was added to the above solution containing the tripeptide and the mixture, after stirring for 48 hr, was concentrated. The product was isolated by procedure B. Recrystallization from MeOH and ether afforded the protected tetrapeptide (12.14 g, 81%), m.p. 108-109°, $[\alpha]_D^{22} - 2.2^\circ$ (c 0.5 in DMF), Rf_1 0.61 (Found: C, 47.90; H, 4.91; N, 11.69. $C_{43}H_{54}N_9O_{15}Cl_3S$ requires C, 48.02; H, 5.06; N, 11.72%).

Z(OMe)-Cys(MBzl)-Arg(MBS)-Glu(OBzl)-Thr-Gly-NHNH-Troc. —————

———— Z(OMe)-Arg(MBS)-Glu(OBzl)-Thr-Gly-NHNH-Troc (5.38 g, 5 mmol) was treated with TFA-anisole (11 ml-2.7 ml) as usual and dry ether was added. The resulting powder was collected by filtration and dissolved in DMF (50 ml) together with Et_3N (1.4 ml, 10 mmol) and Z(OMe)-Cys(MBzl)-ONP (2.63 g, 5 mmol). After stirring for 48 hr, the solution was concentrated and the product was isolated by procedure B. Recrystallization from MeOH and ether afforded the protected pentapeptide (5.76 g, 89%), m.p. 103-105°, $[\alpha]_D^{22} - 9.2^\circ$ (c 0.4 in DMF), Rf_1 0.60 (Found: C, 49.89; H, 5.08; N, 10.83. $C_{54}H_{67}N_{10}O_{17}Cl_3S_2$ requires C, 49.94; H, 5.20; N, 10.79%).

Z(OMe)-Asp(OBzl)-Cys(MBzl)-Arg(MBS)-Glu(OBzl)-Thr-Gly-NHNH-Troc [10]. ————— The above protected pentapeptide (6.49 g, 5 mmol) was treated with TFA-anisole (13 ml-3.2 ml) as usual, then dry ether was added. The resulting powder was dissolved in DMF (35 ml) together with Et₃N (1.4 ml, 10 mmol) and Z(OMe)-Asp(OBzl)-ONP (2.80 g, 5.5 mmol). After stirring for 48 hr, the solution was concentrated and the product was isolated by procedure B. Recrystallization from MeOH and ether afforded the protected hexapeptide (6.25 g, 83%), m.p. 140-142°, $[\alpha]_D^{22} - 10.4^\circ$ (c 0.5 in DMF), Rf₁ 0.64 (Found: C, 51.64; H, 5.29; N, 10.07. C₆₅H₇₈N₁₁O₂₀Cl₃S₂ requires C, 51.91; H, 5.23; N, 10.25%).

Z(OMe)-Asp(OBzl)-Cys(MBzl)-Arg(MBS)-Glu(OBzl)-Thr-Gly-NHNH₂, Z(OMe)-(RNase 83-88)-NHNH₂ ————— The above protected hexapeptide (9.02 g, 6 mmol) dissolved in AcOH (90 ml) was treated with Zn powder (4.8 g, 12 equiv.) for 48 hr. The filtered solution was concentrated and the residue was treated with a saturated solution of EDTA and 5% Na₂CO₃. The resulting gelatinous mass was washed with H₂O and recrystallized from MeOH to give the protected hexapeptide hydrazide (5.40 g, 68%), m.p. 130-133°, $[\alpha]_D^{22} - 8.0^\circ$ (c 0.4 in DMSO), Rf₁ 0.55. Amino acid analysis: Asp 1.04, Arg 1.09, Glu 1.05, Thr 1.01, Gly 1.00, average recovery 90% (Found: C, 55.55; H, 5.85; N, 11.35. C₆₂H₇₇N₁₃O₁₈S₂·H₂O requires C, 55.30; H, 5.91; N, 11.44%).

Z(OMe)-Ser-Ile-Thr-OMe. ————— Z-Ile-Thr-OMe⁹²⁾ (30.40 g, 80 mmol) in THF (200 ml) was hydrogenated over a Pd catalyst in the presence of 1N HCl (80 ml, 1 equiv.) for 8 hr. The catalyst was removed by filtration, the filtrate was concentrated and the residue was dissolved in DMF (300 ml) together with Et₃N (22.1 ml, 0.16 mol) and Z(OMe)-Ser-OPCP (45.45 g, 88 mmol). After stirring for 48 hr, the solution was concentrated and the product was isolated by pro-

cedure B. Recrystallization from MeOH and AcOEt afforded the protected tripeptide ester (28.76 g, 72%), m.p. 189-191°, $[\alpha]_D^{23} + 3.6^\circ$ (c 0.8 in DMF), Rf_1 0.43 (Found: C, 55.56; H, 7.17; N, 8.44. $C_{23}H_{35}N_3O_9$ requires C, 55.52; H, 7.09; N, 8.45%).

Z(OMe)-Ser-Ile-Thr-NHNH₂, Z(OMe)-(RNase 80-82)-NHNH₂ [11].

———— The above protected tripeptide ester (10.40 g, 21 mmol) in DMF-MeOH (80 ml-20 ml) was treated with 80% hydrazine hydrate (11 ml, 13 equiv.) overnight. Precipitation of the resulting mass from DMF with MeOH afforded the hydrazide (8.11 g, 78%), m.p. 240-242°, $[\alpha]_D^{22} - 1.3^\circ$ (c 0.8 in DMSO), Rf_1 0.52. Amino acid analysis: Ser 0.74, Ile 1.00, Thr 0.91, average recovery 77% (Found: C, 52.88; H, 6.92; N, 13.82. $C_{22}H_{35}N_5O_8$ requires C, 53.11; H, 7.09; N, 14.08%).

Z(OMe)-Thr-Met-OMe. ————— Z(OMe)-Thr-OPCP (79.65 g, 0.15 mol) was added to a stirred solution of H-Met-OMe (prepared from 30.0 g, 0.15 mol of the hydrochloride with 20.7 ml, 0.15 mol of Et₃N) in DMF (300 ml). After 48 hr, the solution was concentrated and the residue was purified by procedure B followed by recrystallization from MeOH and AcOEt to give the protected dipeptide ester (37.23 g, 58%), m.p. 111-112°, $[\alpha]_D^{22} - 5.2^\circ$ (c 1.0 in DMF), Rf_1 0.77 (Found: C, 52.99; H, 6.32; N, 6.28. $C_{19}H_{28}N_2O_7$ requires C, 53.25; H, 6.59; N, 6.54%).

Z(OMe)-Ser-Thr-Met-OMe. ————— Z(OMe)-Thr-Met-OMe (38.02 g, 89 mmol) was treated with TFA-anisole (50 ml-18 ml) as usual, then excess TFA was removed by evaporation. The oily residue was washed with n-hexane, dried over KOH pellets in vacuo for 3 hr and dissolved in DMF (300 ml) together with Et₃N (24.8 ml, 0.18 mol) and Z(OMe)-Ser-OPCP (46.03 g, 89 mmol). After stirring for 48 hr, the solution was concentrated and the residue was purified by procedure

B followed by recrystallization from MeOH and AcOEt to give the protected tripeptide ester (37.71 g, 82%), m.p. 148-150°, $[\alpha]_D^{23}$ - 6.6° (c 1.1 in DMF), Rf_1 0.77 (Found: C, 49.29; H, 6.50; N, 7.69. $C_{22}H_{33}N_3O_9S \cdot H_2O$ requires C, 49.52; H, 6.61; N, 7.88%).

Z(OMe)-Ser-Thr-Met(O)-NHNH₂, Z(OMe)-(RNase 77-79)-NHNH₂ [12].

———— Z(OMe)-Ser-Thr-Met-OMe (15.04 g, 29 mmol) in DMF-MeOH (12 ml-60 ml) was treated with $HAuCl_4 \cdot 4H_2O$ (13.22 g, 32 mmol) in H_2O in the presence of $NaHCO_3$ (7.36 g, 3 equiv.) at 40° for 3 hr. After filtration, the filtrate was concentrated and the residue was treated with AcOEt. Recrystallization of the resulting powder three times from MeOH and EtOH afforded the sulphoxide contaminated with a small amount of inorganic salts (11.49 g, 74%), m.p. 161-163°, Rf_1 0.57. This sulphoxide (22 mmol) in MeOH (110 ml) was treated with 80% hydrazine hydrate (6.9 ml, 5equiv.) overnight. Precipitation of the resulting mass twice from DMF with MeOH gave the protected tripeptide hydrazide (10.89 g, 95%), m.p. 183-189°, $[\alpha]_D^{23}$ + 3.5° (c 0.9 in DMSO), Rf_1 0.47. Amino acid analysis : Ser 0.91, Thr 1.00, Met 0.80, average recovery 80% (Found: C, 46.90; H, 6.49; N, 13.06. $C_{21}H_{33}N_5O_9S \cdot 1/2 H_2O$ requires C, 46.65; H, 6.34; N, 12.96%).

Z(OMe)-Gln-Ser-Tyr-OMe. ————— Z-Ser-Tyr-OMe³⁵ (29.24 g, 70 mmol) in THF (240 ml) was hydrogenated over a Pd catalyst in the presence of 1N HCl (70 ml, 1 equiv.) for 8 hr. The catalyst was removed by filtration, the filtrate was concentrated and the residue was dissolved in DMF (200 ml). To this ice-chilled solution, Et_3N (9.7 ml, 70 mmol), Z(OMe)-Gln-OH (21.72 g, 70 mmol), HOBT (9.45 g, 70 mmol) and DCC (15.86 g, 77 mmol) were successively added. The mixture was stirred in an ice-bath for 48 hr and the filtered solution was concentrated. Treatment of the residue with AcOEt afforded a powder, which was purified by procedure B followed by recrystallization from MeOH to afford the protected tripeptide ester (27.44g,

68%), m.p. 206-209°, $[\alpha]_D^{23} + 8.8^\circ$ (c 0.9 in DMF), R_{f1} 0.64. No CN bond vibration in the IR spectrum. (Found: C, 56.48; H, 5.97; N, 9.72. $C_{27}H_{34}N_4O_{10}$ requires C, 56.44; H, 5.96; N, 9.75%).

Z(OMe)-Gln-Ser-Tyr-NHNH₂, Z(OMe)-(RNase 74-76)-NHNH₂ [13].
 ——— Z(OMe)-Gln-Ser-Tyr-OMe (20.20 g, 35 mmol) in DMF-MeOH (40 ml-80 ml) was treated with 80% hydrazine hydrate (14 ml, 6.4 equiv.) overnight. The resulting mass was precipitated from MeOH to yield the hydrazide (17.72 g, 88%), m.p. 231-234°, $[\alpha]_D^{22} + 5.5^\circ$ (c 0.9 in DMSO), R_{f1} 0.21. Amino acid analysis: Glu 1.00, Ser 0.86, Tyr 0.95, average recovery 99% (Found: C, 54.37; H, 5.82; N, 14.40. $C_{26}H_{34}N_6O_9$ requires C, 54.35; H, 5.96; N, 14.63%).

* Z(OMe)-Cys(MBzl)-Tyr-OMe. ——— DCC (22.66 g, 0.11 mol) was added to a mixture of Z(OMe)-Cys(MBzl)-OH (40.54 g, 0.1 mol) and H-Tyr-OMe (prepared from 23.17 g, 0.1 mol of the hydrochloride with 13.8 ml, 0.1 mol of Et₃N) in DMF (400 ml) and the mixture was stirred for 48 hr. The solution was filtered, the filtrate was concentrated and the residue was purified by procedure A followed by recrystallization from MeOH to afford the protected tripeptide ester (45.51 g, 78%), m.p. 141-143°, $[\alpha]_D^{23} -26.8^\circ$ (c 1.0 in DMF), R_{f1} 0.80 (Found: C, 61.82; H, 6.06; N, 4.77. $C_{30}H_{34}N_2O_8S$ requires C, 61.84; H, 5.88; N, 4.80%).

Z(OMe)-Asn-Cys(MBzl)-Tyr-OMe. ——— Z(OMe)-Cys(MBzl)-Tyr-OMe (58.62 g, 0.1 mol) was treated with TFA-anisole (80 ml-30 ml) as usual, then excess TFA was removed by evaporation. The oily residue was washed with n-hexane, dried over KOH pellets in vacuo for 3 hr and dissolved in DMF (400 ml) together with Et₃N (13.8 ml, 0.1 mol) and Z(OMe)-Asn-ONP (41.73 g, 0.1 mol). After stirring for 48 hr, the solution was concentrated. Treatment of the residue with AcOEt afforded a fine powder, which was purified by procedure B

followed by recrystallization from dioxane and MeOH to give the protected tripeptide ester (45.79 g, 66%), m.p. 178-181°, $[\alpha]_D^{23}$ - 27.5° (c 0.7 in DMF), Rf_1 0.65 (Found: C, 57.97; H, 5.86; N, 7.90. $C_{34}H_{40}N_4O_{10}S \cdot 1/2 H_2O$ requires C, 57.86; H, 5.86; N, 7.94%).

Z(OMe)-Asn-Cys(MBzl)-Tyr-NHNH₂, Z(OMe)-(RNase 71-73)-NHNH₂ [14]. ————— Z(OMe)-Asn-Cys(MBzl)-Tyr-OMe (6.97 g, 10 mmol) dissolved in DMF (60 ml) was treated with 80% hydrazine hydrate (3.2 ml, 5 equiv.) overnight. The resulting gelatinous mass was precipitated from DMF with MeOH to yield the hydrazide (5.77 g, 83%), m.p. 243-247°, $[\alpha]_D^{23}$ - 19.8° (c 1.1 in DMSO), Rf_1 0.42. Amino acid analysis: Asp 1.00, Tyr 0.98, average recovery 81% (Found: C, 56.63; H, 6.06; N, 11.94. $C_{33}H_{40}N_6O_9S$ requires C, 56.88; H, 5.79; N, 12.06%).

Z(OMe)-Gln-Thr-OMe. ————— DCC (20.60 g, 0.1 mol) was added to an ice-chilled solution of Z(OMe)-Gln-OH (31.03 g, 0.1 mol), HOBT (13.40 g, 0.1 mol) and H-Thr-OMe (prepared from 16.81 g, 0.1 mol of the hydrochloride with 13.8 ml, 0.1 mol of Et₃N) in DMF (300 ml). After stirring for 48 hr, the solution was filtered, the filtrate was concentrated and the residue was treated with ether. The resulting powder was purified by procedure B followed by recrystallization from MeOH to yield the dipeptide ester (17.44 g, 41%), m.p. 175-177°, $[\alpha]_D^{22}$ + 4.0° (c 0.5 in DMF), Rf_1 0.76 (Found: C, 53.70; H, 6.36; N, 10.00. $C_{19}H_{27}N_3O_8$ requires C, 53.64; H, 6.40; N, 9.88%).

Z(OMe)-Gln-Thr-NHNH₂, Z(OMe)-(RNase 69-70)-NHNH₂ [15]. ————— Z(OMe)-Gln-Thr-OMe (16.0 g, 38 mmol) dissolved in DMF-MeOH (100 ml-100 ml) was treated with 80% hydrazine hydrate (12.2 ml, 5 equiv.). The gelatinous mass formed on standing overnight was precipitated from DMF with MeOH to yield the hydrazide (13.20 g,

83%), m.p. 234-237°, $[\alpha]_D^{22} + 5.2^\circ$ (c 0.6 in DMSO), Rf_1 0.44. Amino acid analysis: Glu 1.00, Thr 0.86, average recovery 82% (Found: C, 50.69; H, 6.32; N, 16.54. $C_{18}H_{27}N_5O_7$ requires C, 50.81; H, 6.40; N, 16.46%).

Z(OMe)-Asp(OBzl)-Cys(MBzl)-Arg(MBS)-Glu(OBzl)-Thr-Gly-Ser-Ser-Lys(Z)-Tyr-Pro-Asn-Cys(MBzl)-Ala-Tyr-Lys(Z)-Thr-Thr-Gln-Ala-Asn-Lys(Z)-His-Ile-Ile-Val-Ala-Cys(MBzl)-Glu(OBzl)-Gly-Asn-Pro-Tyr-Val-Pro-Val-His-Phe-Asp(OBzl)-Ala-Ser-Val-OBzl, [Abbreviated as Z(OMe)-(RNase 83-124)-OBzl or Z(OMe)-Asp(OBzl)-Cys(MBzl)-Arg(MBS)-Glu(OBzl)-Thr-Gly-(89-124)-OBzl].

Z(OMe)-(RNase 89-124)-OBzl (14.38 g, 2.79 mmol) was treated with TFA-anisole (60 ml-15 ml) as usual, then dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets in vacuo for 3 hr and dissolved in DMSO-DMF (1:1, 100 ml) containing Et_3N (1.16 ml, 8.37 mmol). To this ice-chilled solution, the azide (prepared from 9.37 g, 6.98 mmol of Z(OMe)-Asp(OBzl)-Cys(MBzl)-Arg(MBS)-Glu(OBzl)-Thr-Gly-NHNH₂) in DMF (50 ml) and Et_3N (0.96 ml, 6.96 mmol) were added and the mixture was stirred at 4° for 48 hr. The solvent was evaporated and the product was purified by procedure B followed by precipitations from DMSO with MeOH four times to give the protected dotetracontapeptide ester; yield 14.35 g (81%), m.p. 273° dec., $[\alpha]_D^{23} -29.5^\circ$ (c 1.0 in DMSO), Rf_3 0.69 (Found: C, 56.59; H, 6.39; N, 12.64. $C_{300}H_{394}N_{56}O_{79}S_4 \cdot 9H_2O$ requires C, 56.84; H, 6.55; N, 12.37%).

Z(OMe)-(RNase 80-124)-OBzl [Z(OMe)-Ser-Ile-Thr-(83-124)-OBzl].

————— Z(OMe)-(RNase 83-124)-OBzl (11.16 g, 1.76 mmol) was treated with TFA-anisole (50 ml-10 ml) and the N^α -deprotected peptide, isolated as mentioned above, was dissolved in DMSO-DMF (1:1, 100 ml) containing Et_3N (0.73 ml, 5.28 mmol). To this ice-chilled solution, the azide (prepared from 2.19 g, 4.40 mmol of Z(OMe)-Ser-

Ile-Thr-NHNH₂) in DMF (20 ml) and Et₃N (0.61 ml, 4.40 mmol) were added. After 48 hr, the solvent was evaporated off and the product was purified by procedure B followed by three times precipitations from DMSO with MeOH to yield the protected pentatetracontapeptide ester (10.23 g, 87%), m.p. 252° dec., $[\alpha]_D^{23}$ -32.6° (c 0.9 in DMSO), R_f₃ 0.84 (Found: C, 55.79; H, 6.57; N, 12.72. C₃₁₃H₄₁₇N₅₉O₈₄S₄.13 H₂O requires C, 56.00; H, 6.65; N, 12.31%).

Z(OMe)-(RNase 77-124)-OBzl [Z(OMe)-Ser-Thr-Met(O)-(80-124)-OBzl]. ————— Z(OMe)-(RNase 80-124)-OBzl (9.41 g, 1.40 mmol) was treated with TFA-anisole (50 ml-10 ml) and the N^α-deprotected peptide, isolated as mentioned above, was dissolved in DMSO-DMF (2:1, 100 ml) containing Et₃N (0.58 ml, 4.20 mmol). To this ice-chilled solution, the azide (prepared from 1.89 g, 3.50 mmol of Z(OMe)-Ser-Thr-Met(O)-NHNH₂) in DMF (15 ml) and Et₃N (0.48 ml, 3.50 mmol) were added. After 48 hr, the solvent was evaporated and the residue was purified by procedure B followed by precipitations from DMSO with MeOH (twice) to yield the protected octatetracontapeptide ester (9.44 g, 96%), m.p. 257° dec., $[\alpha]_D^{23}$ - 23.8° (c 0.7 in DMSO), R_f₃ 0.86 (Found: c, 55.00; H, 6.45; N, 12.91. C₃₂₅H₄₃₈N₆₂O₉₀S₅.13 H₂O requires C, 55.38; H, 6.45; N, 12.32%).

Z(OMe)-(RNase 74-124)-OBzl [Z(OMe)-Gln-Ser-Tyr-(77-124)-OBzl]. ————— Z(OMe)-(RNase 77-124)-OBzl (9.44 g, 1.34 mmol) was treated with TFA-anisole (50 ml-10 ml) and the N^α-deprotected peptide, isolated as mentioned above, was dissolved in DMSO-DMF (1:1, 100 ml) containing Et₃N (0.55 ml, 4.02 mmol). To this ice-chilled solution, the azide (prepared from 2.31 g, 4.02 mmol of Z(OMe)-Gln-Ser-Tyr-NHNH₂) in DMF (20 ml) and Et₃N (0.55 ml, 4.02 mmol) were added. After 48 hr, the solvent was evaporated and the residue was purified by procedure B followed by precipitations from DMSO with MeOH (five times) to yield the protected henpentacontapeptide ester

(9.25 g, 92%), m.p. 246°dec., $[\alpha]_D^{23} - 28.5^\circ$ (c 0.8 in DMSO), Rf_3 0.81, Rf_5 0.43 (Found: C, 54.51; H, 6.34; N, 12.50. $C_{325}H_{438}N_{66}O_{96}S_5 \cdot 18 H_2O$ requires C, 54.64; H, 6.41; N, 12.30%).

Z(OMe)-(RNase 71-124)-OBzl [Z(OMe)-Asn-Cys(MBzl)-Tyr-(74-124)-OBzl]. ————— Z(OMe)-(RNase 74-124)-OBzl (9.25 g, 1.23 mmol) was treated with TFA-anisole (50 ml-10 ml) and the N^α -deprotected peptide, isolated as mentioned above, was dissolved in DMF-NMP (1:2, 100 ml) containing Et_3N (0.41 ml, 3.69 mmol). To this ice-chilled solution, the azide (prepared from 2.15 g, 3.08 mmol of Z(OMe)-Asn-Cys(MBzl)-Tyr-NHNH₂) in DMF (15 ml) and Et_3N (0.43 ml, 3.08 mmol) were added. After stirring for 48 hr, the solution was concentrated and the residue was purified by procedure B followed by precipitations from DMSO with MeOH (three times) to yield the protected tetrapentacontapeptide ester (9.04 g, 92%), m.p. 253° dec., $[\alpha]_D^{23} - 26.8^\circ$ (c 0.9 in DMSO), Rf_3 0.78 (Found: C, 54.71; H, 6.33; N, 12.67. $C_{366}H_{488}N_{70}O_{102}S_6 \cdot 17 H_2O$ requires C, 54.95; H, 6.36; N, 12.26%).

Z(OMe)-(RNase 69-124)-OBzl [Z(OMe)-Gln-Thr-(71-124)-OBzl]. ————— Z(OMe)-(RNase 71-124)-OBzl (9.04 g, 1.18 mmol) was treated with TFA-anisole (45 ml-9 ml) and the N^α -deprotected peptide isolated as mentioned above was dissolved in DMSO-DMF (2:1, 90 ml) containing Et_3N (0.49 ml, 3.54 mmol). To this ice-chilled solution, the azide (prepared from 1.26 g, 2.95 mmol) of Z(OMe)-Gln-Thr-NHNH₂ in DMF (10 ml) and Et_3N (0.41 ml, 2.95 mmol) were added. After 48 hr, the solvent was removed by evaporation and the residue was purified by procedure B followed by precipitations from DMSO with MeOH (three times) to yield the protected hexapentacontapeptide ester (8.74 g, 94%), m.p. 238° dec., $[\alpha]_D^{23} - 28.4^\circ$ (c 1.1 in DMSO), Rf_3 0.72 (Found: C, 52.76; H, 6.25; N, 12.22. $C_{375}H_{503}N_{73}O_{106}S_6 \cdot 32 H_2O$ requires C, 53.00; H, 6.73; N, 12.03%).

Purification of the crude sample of Z(OMe)-(RNase 69-124)-OBzl by gel-filtration on Sephacryl S-200. —————

The first batch of Z(OMe)-(RNase 69-124)-OBzl prepared as outlined above gave somewhat higher recovery of Asp, Thr and Glu than those predicted by theory (Table 3'). Such a tendency was also noted in the intermediates, Z(OMe)-(RNase 71-124)-OBzl and Z(OMe)-(RNase 74-124)-OBzl as well, where newly incorporated amino acid ratios were somewhat higher than expected. The impure preparation of Z(OMe)-(RNase 69-124)-OBzl (100 mg each) was submitted to gel-filtration on various supports (2.8 x 130 cm) with DMSO, 5% H₂O-DMSO or NMP as eluents: 1. LH-20 (DMSO), 2. Sephadex G-50 (5% H₂O-DMSO), 3. Sephadex G-100 (5% H₂O-DMSO), 4. Sepharose CL-6B (5% H₂O-DMSO), 5. Sephacryl S-200 (5% H₂O-NMP), 6. Biogel P-2 (for the N^α-deprotected peptide with 66% AcOH as eluent). Individual fractions (9.5 ml each) were collected and absorption at 275 nm was measured. No separation was obtained in experiments 1,2,3 and 6. Gel-filtration on Sepharose CL-6B gave some separation (Peak 1, tube Nos. 35-50, 46 mg; Peak 2, tube Nos. 56 -70, 14 mg) and on Sephacryl S-200, a better separation was achieved (Peak 1, tube Nos. 24-31, 58 mg; Peak 2, tube Nos. 36-45, 7 mg) as shown in Fig. 2. Purity of other batches of Z(OMe)-(RNase 69-124)-OBzl was confirmed by gel-filtration on Sephacryl S-200.

Section 4. Synthesis of the Protected Tetraoctacontapeptide Ester (positions 41-124)

Z(OMe)-Asn-Gly-OH. ————— Z(OMe)-Asn-ONP (20.85 g, 50 mmol) dissolved in DMF (150 ml) was added to a stirred solution of Gly (11.26 g, 0.15 mol) and Et₃N (20.7 ml, 0.15 mol) in H₂O (80 ml). After 48 hr, the solution was concentrated and the residue was treated with ether and 5% citric acid. The resulting powder was washed three times with 5% citric acid and H₂O and recrystallized from MeOH and AcOEt to afford the protected tripeptide (6.70 g, 38%), m.p. 165-167°, $[\alpha]_D^{23} - 1.4^\circ$ (c 0.7 in DMF), Rf₂ 0.07 (Found: C, 50.95; H, 5.43; N, 11.92. C₁₅H₁₉N₃O₇ requires C, 50.99; H, 5.42; N, 11.89%).

Z(OMe)-Lys(Z)-Asn-Gly-OH. ————— Z(OMe)-Asn-Gly-OH (10.59 g, 30 mmol) was treated with TFA-anisole (21 ml-5.3 ml) as usual, then dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets in vacuo for 3 hr and dissolved in DMF (150 ml) together with Et₃N (8.3 ml, 60 mmol) and Z(OMe)-Lys(Z)-OQCl (20.59 g, 33 mmol). After stirring for 48 hr, the solution was concentrated and the residue was treated with ether and 5% citric acid. The resulting powder was washed as mentioned above and recrystallized from MeOH and ether to afford the protected tripeptide (4.22 g, 23%), m.p. 198-200°, $[\alpha]_D^{22} - 6.1^\circ$ (c 0.5 in DMF), Rf₂ 0.15 (Found: C, 56.37; H, 6.09; N, 11.41. C₂₉H₃₇N₅O₁₀ requires C, 56.57; H, 6.06; N, 11.38%).

Z(OMe)-Asn-Gly-NHNH-Troc. ————— Z(OMe)-Gly-NHNH-Troc⁹⁷⁾ (20.60 g, 48 mmol) was treated with TFA-anisole (41 ml -10 ml) as usual, then excess TFA was removed by evaporation. The oily residue was washed with n-hexane, dried over KOH pellets in vacuo for 3 hr and dissolved in DMF (100 ml) together with Et₃N (12.1 ml, 88 mmol)

and Z(OMe)-Asn-ONP (16.68 g, 40 mmol). After stirring for 48 hr, the solution was concentrated and the residue was purified by the procedure B followed by recrystallization from MeOH to afford the protected dipeptide (13.61 g, 52%), m.p. 175-178°, $[\alpha]_D^{23} - 6.9^\circ$ (c 0.6 in DMF), Rf_2 0.15 (Found: C, 39.77; H, 4.20; N, 12.69. $C_{18}H_{22}N_5O_8Cl_3$ requires C, 39.83; H, 4.09; N, 12.90%).

Z(OMe)-Lys(Z)-Asn-Gly-NHNH-Troc. ————— (a) Z(OMe)-Asn-Gly-NHNH-Troc (10.85 g, 20 mmol) was treated with TFA-anisole (22 ml- 5.4 ml) as usual, then dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets in vacuo for 3 hr and dissolved in DMF (80 ml) together with Et_3N (5.5 ml, 40 mmol) and Z(OMe)-Lys(Z)-OQCl (12.85 g, 20 mmol). After stirring for 48 hr, the solution was concentrated and the residue was purified by procedure B followed by recrystallization from MeOH to afford the protected tripeptide (13.73 g, 85%), m.p. 190-193°, $[\alpha]_D^{23} - 6.8^\circ$ (c 1.0 in DMF), Rf_1 0.51, Rf_2 0.20 (Found: C, 47.91; H, 4.85; N, 12.31. $C_{32}H_{40}N_7O_{11}Cl_3$ requires C, 47.74; H, 5.01; N, 12.18 %).

(b) DCC (1.68 g, 8.2 mmol) was added to a mixture of Z(OMe)-Lys(Z)-Asn-Gly-OH (4.18 g, 6.8 mmol), HOBT (1.11 g, 8.2 mmol) and Troc-NHNH₂ (1.70 g, 8.2 mmol) in DMF (20 ml). After stirring for 24 hr, the solution was filtered, the filtrate was concentrated and the product was isolated as stated above; yield 4.74 g (87%), m.p. 190-193°, $[\alpha]_D^{25} - 6.5^\circ$ (c 0.7 in DMF).

Z(OMe)-Cys(MBzl)-Lys(Z)-Asn-Gly-NHNH-Troc [16]. ————— Z(OMe)-Lys(Z)-Asn-Gly-NHNH-Troc (12.08 g, 15 mmol) was treated with TFA-anisole (24 ml- 6 ml) as usual, then dry ether was added. The resulting powder, isolated as mentioned above, was dissolved in DMF (100 ml) together with Et_3N (4.1 ml, 30 mmol) and Z(OMe)-Cys(MBzl)-ONP (9.48 g, 18 mmol). After stirring for 48 hr, the solution was

concentrated and the residue was purified by procedure B. Precipitation from DMF with MeOH afforded the protected tetrapeptide (11.91 g, 77%), m.p. 213-216°, $[\alpha]_D^{23} - 17.7^\circ$ (c 0.5 in DMF), $Rf_1 0.44$ (Found: C, 50.25; H, 5.20; N, 10.82. $C_{43}H_{53}N_8O_{13}SCl_3$ requires C, 50.22; H, 5.20; N, 10.90%).

Z(OMe)-Cys(MBzl)-Lys(Z)-Asn-Gly-NHNH₂, Z(OMe)-(RNase 65-68)-NHNH₂. ————— Zn powder (6.53 g, 10 equiv.) was added to a stirred solution of Z(OMe)-Cys(MBzl)-Lys(Z)-Asn-Gly-NHNH-Troc (10.28 g, 10 mmol) in DMF-AcOH (1:1, 200 ml). After 72 hr, the solution was filtered and the filtrate was concentrated in vacuo at 30°. Treatment of the residue with a saturated solution of EDTA afforded a gelatinous mass, which was washed with H₂O and precipitated from DMF with MeOH to yield the protected tetrapeptide hydrazide (6.27 g, 74%), m.p. 212-215°, $[\alpha]_D^{23} - 12.8^\circ$ (c 0.9 in DMSO), $Rf_1 0.53$. Amino acid analysis: Lys 1.00, Asp 1.18, Gly 1.12, average recovery 99% (Found: C, 56.13; H, 6.06; N, 12.89. $C_{40}H_{52}N_8O_{11}S$ requires C, 56.32; H, 6.15; N, 13.14%).

Z(OMe)-Asn-Val-Ala-OMe. ————— Z(OMe)-Val-Ala-OMe (15.50 g, 42 mmol) was treated with TFA-anisole (31 ml-7.7 ml) as usual, then excess TFA was removed by evaporation. The oily residue was washed with n-hexane, dried over KOH pellets in vacuo for 2 hr and dissolved in DMF (80 ml) together with Et₃N (11.9 ml, 86 mmol) and Z(OMe)-Asn-ONP (17.93 g, 43 mmol). After stirring for 48 hr, the solution was concentrated and the product was purified by procedure B followed by precipitation from DMF with MeOH to yield the protected tripeptide ester (16.50 g, 82%), m.p. 243-245°, $[\alpha]_D^{23} - 14.6^\circ$ (c 0.5 in DMF), $Rf_2 0.35$ (Found: C, 54.76; H, 6.58; N, 11.50. $C_{22}H_{32}N_4O_8$ requires C, 54.99; H, 6.71; N, 11.66%).

Z(OMe)-Asn-Val-Ala-NHNH₂, Z(OMe)-(RNase 62-64)-NHNH₂ [17].

————— Z(OMe)-Asn-Val-Ala-OMe (16.50 g, 34 mmol) in DMF (160 ml) was treated with 80% hydrazine hydrate (21 ml, 10 equiv.) overnight and MeOH (160 ml) was added. The resulting mass was precipitated from DMF with MeOH to give the protected tripeptide hydrazide (15.20 g, 93%), m.p. 246-248°, $[\alpha]_D^{23} - 14.5^\circ$ (c 0.3 in DMSO), Rf_1 0.46. Amino acid analysis: Asp 1.04, Val 1.00, Ala 0.94, average recovery 92% (Found: C, 52.69; H, 6.77; N, 17.46. $C_{21}H_{32}N_6O_7$ requires C, 52.49; H, 6.71; N, 17.49%).

Z(OMe)-Gln-Lys(Z)-OH. ————— Z(OMe)-Gln-ONP (21.57 g, 50 mmol) dissolved in pyridine (70 ml) was mixed with a solution of Lys(Z) (14.02 g, 50 mmol) and Et_3N (13.8 ml, 0.1 mol) in H_2O (70 ml). After stirring for 48 hr, the solution was concentrated and the residue was treated with 5% citric acid and ether. The resulting powder was purified by washing 5% citric acid and H_2O followed by recrystallization from MeOH and AcOEt to yield the protected dipeptide (16.0 g, 56%), m.p. 172-174° $[\alpha]_D^{23} + 4.9^\circ$ (c 0.7 in DMF), Rf_2 0.15 (Found: C, 58.68; H, 6.28; N, 9.72. $C_{28}H_{36}N_4O_9$ requires C, 58.73; H, 6.34; N, 9.79%).

Z(OMe)-Cys(MBzl)-Ser-OMe. ————— Z(OMe)-Cys(MBzl)-ONP (21.10 g, 40 mmol) in DMF (50 ml) and Et_3N (5.5 ml, 40 mmol) were added to a solution of H-Ser-OMe (prepared from 7.50 g, 48 mmol of the hydrochloride with 6.6 ml, 48 mmol of Et_3N) in DMF (50 ml). After stirring for 48 hr, the solution was concentrated and the residue was purified by procedure B followed by recrystallization from THF and MeOH to afford the protected dipeptide ester (15.50 g, 76%), m.p. 134-135°, $[\alpha]_D^{23} - 28.7^\circ$ (c 1.2 in DMF), Rf_1 0.58 (Found: C, 57.12; H, 6.07; N, 5.64. $C_{24}H_{30}N_2O_8S$ requires C, 56.90; H, 5.97; N, 5.53%).

Z(OMe)-Cys(MBzl)-Ser-NHNH₂. ————— Z(OMe)-Cys(MBzl)-

Ser-OMe (15.20 g, 30 mmol) in DMF (60 ml) was treated with 80% hydrazine hydrate (19 ml, 10 equiv.) and the gelatinous mass formed on standing overnight was precipitated from DMF with MeOH to give the hydrazide (13.20 g, 87%), m.p. 193-194°, $[\alpha]_D^{23} - 4.8^\circ$ (c 1.4 in DMSO), Rf_1 0.53 (Found: C, 54.66; H, 5.95; N, 10.76. $C_{23}H_{30}N_4O_7S$ requires C, 54.53; H, 5.97; N, 11.06%).

Z(OMe)-Cys(MBzl)-Ser-Gln-Lys(Z)-OH. ————— Z(OMe)-Gln-Lys(Z)-OH (7.44 g, 13 mmol) was treated with TFA-anisole (14.8 ml-3.7 ml) as usual and the TFA salt, precipitated with dry ether as a fine powder, was collected by filtration, dried over KOH pellets in vacuo for 3 hr and dissolved in DMF (40 ml) containing Et_3N (3.6 ml, 26 mmol). To this ice-chilled solution, the azide (prepared from 7.89 g, 16 mmol of Z(OMe)-Cys(MBzl)-Ser-NHNH₂) in DMF (50 ml) and Et_3N (2.2 ml, 16 mmol) were added. After stirring for 48 hr, the solution was concentrated and the residue was purified by washing with 5% citric acid and H₂O followed by recrystallization from MeOH and AcOEt to afford the protected tetrapeptide (7.41 g, 65%), m.p. 168-169°, $[\alpha]_D^{23} - 8.8^\circ$ (c 0.8 in DMF), Rf_2 0.18 (Found: C, 57.37; H, 6.29; N, 9.49. $C_{42}H_{54}N_6O_{13}S$ requires C, 57.13; H, 6.16; N, 9.52 %).

Z(OMe)-Val-Cys(MBzl)-Ser-Gln-Lys(Z)-OH. ————— The above protected tetrapeptide (13.65 g, 15 mmol) was treated with TFA-anisole (27 ml-6.8 ml) as usual, then dry ether was added. The resulting powder, isolated as mentioned above, was dissolved in DMF (70 ml) together with Et_3N (4.1 ml, 30 mmol) and Z(OMe)-Val-ONP (6.84 g, 17 mmol). After stirring for 48 hr, the solution was concentrated and the residue was purified by washing with 5% citric acid and H₂O followed by precipitation from DMF with MeOH to yield the protected pentapeptide (13.70 g, 90%), m.p. 203-204°, $[\alpha]_D^{22} - 8.0^\circ$ (c 1.1 in DMF), Rf_1 0.12 (Found: C, 57.39; H, 6.22; N, 10.05.

$C_{47}H_{63}N_7O_{14}S$ requires C, 57.48; H, 6.47; N, 9.98%.

Z(OMe)-Val-Cys(MBzl)-Ser-Gln-Lys(Z)-OMe. _____

An ethereal solution of diazomethane was added to an ice-chilled solution of Z(OMe)-Val-Cys(MBzl)-Ser-Gln-Lys(Z)-OH (13.70 g, 14 mmol) in DMF-MeOH (150 ml-50 ml) till the yellow colour persisted for 30 min. After addition of a few drops of AcOH, the solvent was evaporated and the resulting mass was precipitated from DMF with MeOH to give the pentapeptide ester (11.65 g, 84%), m.p. 227-229°, $[\alpha]_D^{23}$ - 8.2° (c 0.4 in DMF), Rf_1 0.58 (Found: C, 57.61; H, 6.53; N, 9.83. $C_{48}H_{65}N_7O_{14}S$ requires C, 57.87; H, 6.58; N, 9.84%).

Z(OMe)-Val-Cys(MBzl)-Ser-Gln-Lys(Z)-NHNH₂, Z(OMe)-(RNase 57-61)-NHNH₂ [18]. _____ Z(OMe)-Val-Cys(MBzl)-Ser-Gln-Lys(Z)-OMe (11.60 g, 12 mmol) in DMF (100 ml) was treated with 80% hydrazine hydrate (7.3 ml, 10 equiv.). The gelatinous mass, which formed on standing overnight, was collected by filtration and precipitated from DMF with MeOH to give the hydrazide (10.50 g, 91%), m.p. 241-243°, $[\alpha]_D^{23}$ - 3.8° (c 1.1 in DMSO), Rf_1 0.46. Amino acid analysis: Val 1.11, Ser 0.94, Glu 1.14, Lys 1.00, average recovery 91% (Found: C, 56.21; H, 6.40; N, 12.47. $C_{47}H_{65}N_9O_{13}S \cdot 1/2 H_2O$ requires C, 56.16; H, 6.62; N, 12.54%).

Z(OMe)-Gln-Ala-NHNH-Troc. _____ Z(OMe)-Ala-NHNH-Troc⁵⁰

(22.08 g, 50 mmol) was treated with TFA-anisole (44 ml-11 ml) as usual, then excess TFA was removed by evaporation. The oily residue was washed with n-hexane, dried over KOH pellets in vacuo for 3 hr and dissolved in DMF (100 ml) together with Et₃N (13.8 ml, 0.1 mol) and Z(OMe)-Gln-ONP (21.57 g, 50 mmol). After stirring for 48 hr, the solution was concentrated and the residue was purified by procedure B followed by recrystallization from MeOH and ether (16.13 g, 57%), m.p. 193-195°, $[\alpha]_D^{23}$ - 9.8° (c 0.7 in DMF), Rf_1 0.55, Rf_2

0.15 (Found: C, 41.87; H, 4.62; N, 12.23. $C_{20}H_{26}N_5O_8Cl_3$ requires C, 42.08; H, 4.59; N, 12.27%).

Z(OMe)-Val-Gln-Ala-NHNH-Troc. ————— Z(OMe)-Gln-Ala-NHNH-Troc (15.96 g, 28 mmol) was treated with TFA-anisole (32 ml-8 ml) as usual, then dry ether was added. The resulting powder was dried over KOH pellets in vacuo for 3 hr and dissolved in DMF (100 ml) together with Et_3N (7.7 ml, 56 mmol) and Z(OMe)-Val-ONP (13.67 g, 34 mmol). After stirring for 48 hr, the solution was concentrated and the residue was purified by procedure B followed by recrystallization from MeOH to yield the protected tripeptide (13.22 g, 70 %), m.p. 201-203°, $[\alpha]_D^{22}$ -33.3° (c 0.5 in DMF), Rf_1 0.52 (Found: C, 44.58; H, 5.09; N, 12.42. $C_{25}H_{35}N_6O_9Cl_3$ requires C, 44.82; H, 5.27; N, 12.55%).

Z(OMe)-Asp(OBzl)-Val-Gln-Ala-NHNH-Troc [19]. ————— Z(OMe)-Val-Gln-Ala-NHNH-Troc (13.39g, 20 mmol) was treated with TFA-anisole (26 ml-6.6 ml) as usual and the N^α -deprotected peptide, isolated as mentioned above, was dissolved in DMF (120 ml) together with Et_3N (5.5 ml, 40 mmol) and Z(OMe)-Asp(OBzl)-ONP (12.19 g, 24 mmol). After stirring for 48 hr, the solution was concentrated and the residue was purified by procedure B followed by precipitation from DMF with MeOH to yield the protected tetrapeptide (11.70 g, 67%), m.p. 200-202°, $[\alpha]_D^{23}$ -17.1° (c 1.1 in DMF), Rf_1 0.56 (Found: C, 49.58; H, 5.14; N, 11.09. $C_{36}H_{46}N_7O_{12}Cl_3$ requires C, 49.40; H, 5.30; N, 11.20%).

Z(OMe)-Asp(OBzl)-Val-Gln-Ala-NHNH₂, Z(OMe)-(RNase 53-56)-NHNH₂. ————— The above protected tetrapeptide (8.0 g, 9 mmol) in DMF-AcOH (50 ml-50 ml) was treated with Zn powder (6.0 g, 10 equiv.) for 72 hr. Some gelatinous mass which formed was brought into solution with a slight warming and the solution was filtered.

The filtrate was concentrated in vacuo at 30° and the residue was treated with a saturated solution of EDTA and 5% NaHCO₃. The resulting mass was collected by filtration, washed with H₂O and precipitated from DMF with MeOH to yield the hydrazide (4.04 g, 63%), m.p. 226°-228°, $[\alpha]_D^{22}$ - 13.7° (c 0.4 in DMSO), Rf₁ 0.53. Amino acid analysis: Asp 1.10, Val 1.00, Glu 1.08, Ala 0.99, average recovery 96% (Found: C, 56.82; H, 6.64; N, 14.01. C₃₃H₄₅N₇O₁₀ requires C, 56.64; H, 6.48; N, 14.01%).

Z(OMe)-Leu-Ala-NHNH-Troc. ————— Z(OMe)-Leu-ONP (22.70 g, 55 mmol) and Et₃N (13.8 ml, 0.1 mol) were added to a solution of H-Ala-NHNH-Troc. TFA salt (prepared from 22.10 g, 50 mmol of the Z(OMe)-derivative as stated above) in DMF (100 ml). After stirring for 48 hr, the solution was concentrated and the residue was purified by procedure A followed by recrystallization from MeOH and ether to yield the protected dipeptide (16.03 g, 58%), m.p. 85-88°, $[\alpha]_D^{22}$ -16.2° (c 0.6 in DMF), Rf₁ 0.85 (Found: C, 45.19; H, 5.20; N, 10.08. C₂₁H₂₉N₄O₇Cl₃ requires C, 45.37; H, 5.26; N, 10.08%).

Z(OMe)-Ser-Leu-Ala-NHNH-Troc. ————— Z(OMe)-Leu-Ala-NHNH-Troc (15.90 g, 29 mmol) was treated with TFA-anisole (32 ml-8 ml) as usual, then dry ether was added. The resulting powder, isolated as mentioned earlier, was dissolved in DMF (80 ml). To this ice-chilled solution, the azide (prepared from 9.70 g, 34 mmol of Z(OMe)-Ser-NHNH₂) in DMF (50 ml) and Et₃N (7.9 ml, 58 mmol) were added. After stirring for 48 hr, the solution was concentrated and the residue was purified by procedure A followed by recrystallization from AcOEt and ether to yield the protected tripeptide (16.15 g, 88%), m.p. 96-99°, $[\alpha]_D^{22}$ - 14.1° (c 0.5 in DMF), Rf₁ 0.67 (Found: C, 44.69; H, 5.27; N, 10.73. C₂₄H₃₄N₅O₉Cl₃ requires C, 44.83; H, 5.33; N, 10.89%).

Z(OMe)-Glu(OBzl)-Ser-Leu-Ala-NHNH-Troc [20]. —————

The above protected tripeptide (16.10 g, 25 mmol) was treated with TFA-anisole (32 ml-8 ml) as usual, then dry ether was added. The resulting powder, isolated as mentioned above, was dissolved in DMF (130 ml) together with Et₃N (6.9 ml, 50 mmol) and Z(OMe)-Glu(OBzl)-ONP (13.10 g, 25 mmol). After stirring for 48 hr, the solution was concentrated and the residue was purified by procedure B followed by recrystallization from MeOH to afford the protected tetrapeptide (17.31 g, 80%), m.p. 182-184°, $[\alpha]_D^{22}$ -10.6° (c 0.7 in DMF), Rf_1 0.64 (Found: C, 49.87; H, 5.26; N, 9.79. C₃₆H₄₇N₆O₁₂Cl₃ requires C, 50.15; H, 5.50; N, 9.75%).

Z(OMe)-Glu(OBzl)-Ser-Leu-Ala-NHNH₂, Z(OMe)-(RNase 49-52)-NHNH₂. —————

The above protected tetrapeptide (12.60 g, 15 mmol) dissolved in DMF-AcOH (60 ml-60 ml) was treated with Zn powder (9.61 g, 10 equiv.) for 72 hr. Some gelatinous mass which formed during the reaction was dissolved with a slight warming. The solution was filtered, the filtrate was concentrated in vacuo at 30° and the residue was treated with a saturated solution of EDTA and 5% NaHCO₃. The resulting mass was washed with H₂O and precipitated from DMF with MeOH to yield the hydrazide (6.50 g, 65%), m.p. 222-224°, $[\alpha]_D^{23}$ - 8.3° (c 0.4 in DMSO), Rf_1 0.65. Amino acid analysis: Glu 1.07, Ser 0.81, Leu 0.91, Ala 1.00, average recovery 95% (Found: C, 57.83; H, 6.78; N, 12.35. C₃₅H₄₆N₆O₁₀ requires C, 57.71; H, 6.75; N, 12.24%).

Z(OMe)-Phe-Val-His-OMe. ————— Z-Val-His-OMe¹⁰¹⁾ (20.12 g,

50 mmol) dissolved in a mixture of THF (150 ml) and 1N HCl (100 ml, 2 equiv.) was hydrogenated over a Pd catalyst for 8 hr, then the catalyst was removed by filtration. The filtrate was concentrated and the residue, after drying over KOH pellets in vacuo for 3 hr, was dissolved in DMF (150 ml) together with (13.8 ml, 0.1 mol) and

Z(OMe)-Phe-OH (16.47 g, 50 mmol). DCC (12.0 g, 58 mmol) was added and the mixture, after stirring for 48 hr, was filtered. The filtrate was concentrated and the residue was treated with ether. The resulting powder was washed with ether and 5% NaHCO₃ and incubated in MeOH (150 ml) containing AcOH (6 ml) at 50° for 4 hr. The solvent was evaporated and the residue was recrystallized from MeOH and AcOEt to afford the protected tripeptide ester (21.42 g, 74%), m.p. 190-192°, $[\alpha]_D^{22}$ - 7.0° (c 0.3 in DMF), Rf_1 0.51 (Found: C, 62.12; H, 6.50; N, 12.10. C₃₀H₃₇N₅O₇ requires C, 62.16; H, 6.43; N, 12.08%).

Z(OMe)-Phe-Val-His-NHNH₂, Z(OMe)-(RNase 46-48)-NHNH₂ [21].

———— The above protected tripeptide ester (5.80 g, 10 mmol) in DMF (50 ml) was treated with 80% hydrazine hydrate (6.3 ml, 10 equiv.) overnight, then the solvent was evaporated. Trituration of the residue with ether followed by recrystallization from MeOH gave the protected tripeptide hydrazide (3.22 g, 56%), m.p. 197-200°, $[\alpha]_D^{22}$ - 2.9° (c 1.0 in DMSO), Rf_1 0.43. Amino acid analysis: Phe 1.05, Val 1.00, His 0.89, average recovery 92% (Found: C, 58.98; H, 6.22; N, 16.59. C₂₉H₃₇N₇O₆·1/2 H₂O requires C, 59.17; H, 6.51; N, 16.66%).

Z(OMe)-Asn-Thr-OMe. ————— Z(OMe)-Asn-ONP (20.50 g, 49 mmol) and Et₃N (6.8 ml, 49 mmol) were added to a stirred solution of H-Thr-OMe (prepared from 9.16 g, 54 mmol of the hydrochloride with 7.5 ml, 54 mmol of Et₃N) in DMF (100 ml). After 48 hr, the solution was concentrated and the residue was purified by procedure B followed by recrystallization from MeOH and AcOEt to give the protected dipeptide ester (12.01 g, 60%), m.p. 168-170°, $[\alpha]_D^{22}$ - 1.5° (c 0.7 in DMF), Rf_1 0.82 (Found: C, 52.26; H, 6.19; N, 9.95. C₁₈H₂₅N₃O₈ requires C, 52.55; H, 6.13; N, 10.21%).

Z(OMe)-Val-Asn-Thr-OMe. ————— Z(OMe)-Asn-Thr-OMe (8.23 g, 20 mmol) was treated with TFA-anisole (16 ml-4 ml) as usual, then dry ether was added. The resulting powder, isolated as mentioned earlier, was dissolved in DMF (50 ml) together with Et₃N (5.5 ml, 40 mmol) and Z(OMe)-Val-ONP (8.85 g, 22 mmol). After stirring for 24 hr, the solution was concentrated and the residue was purified by procedure B followed by precipitation from DMF with MeOH to afford the protected tripeptide ester (6.19 g, 61%), m.p. 229-231°, $[\alpha]_D^{22} - 1.0^\circ$ (c 1.0 in DMF), Rf₁ 0.15 (Found: C, 53.81; H, 6.67; N, 10.93. C₂₃H₃₄N₄O₉ requires C, 54.11; H, 6.71; N, 10.98 %).

Z(OMe)-Lys(Z)-Pro-Val-Asn-Thr-OMe. ————— Z(OMe)-Val-Asn-Thr-OMe (4.09 g, 8.0 mmol) was treated with TFA-anisole (15 ml-2.6 ml) as usual, then dry ether was added. The resulting powder was dissolved in DMF (25 ml) together with Et₃N (2.3 ml, 17 mmol) and Z(OMe)-Lys(Z)-Pro-OPCP¹⁰² (7.10 g, 9.0 mmol). After stirring overnight, the solution was concentrated and the residue was purified by procedure A followed by precipitation from DMF with MeOH to afford the protected pentapeptide ester (4.35 g, 63%), m.p. 193-198°, $[\alpha]_D^{22} - 20.2^\circ$ (c 1.3 in DMF), Rf₁ 0.69 (Found: C, 56.80; H, 6.71; N, 11.07. C₄₂H₅₉N₇O₁₃·H₂O requires C, 56.81; H, 6.92; N, 11.04%).

Z(OMe)-Lys(Z)-Pro-Val-Asn-Thr-NHNH₂, Z(OMe)-(RNase 41-45)-NHNH₂ [22]. ————— Z(OMe)-Lys(Z)-Pro-Val-Asn-Thr-OMe (10.87 g, 12.5 mmol) in DMF (80 ml) was treated with 80% hydrazine hydrate (7.8 ml, 10 equiv.) overnight, then the solvent was evaporated. The residue was treated with MeOH and the resulting mass was precipitated from DMF with MeOH to give the protected pentapeptide hydrazide (6.03 g, 55%), m.p. 192-195°, $[\alpha]_D^{23} - 38.9^\circ$ (c 1.1 in DMSO), Rf₁ 0.45. Amino acid analysis: Lys 1.00, Pro 1.01, Val 0.93, Asp 1.04, Thr 0.88, average recovery 93% (Found: C, 55.56; H, 6.89; N, 11.04).

14.19. $C_{41}H_{59}N_9O_{12} \cdot H_2O$ requires C, 55.45; H, 6.92; N, 14.20%).

Z(OMe)-Cys(MBzl)-Lys(Z)-Asn-Gly-Gln-Thr-Asn-Cys(MBzl)-Tyr-Gln-Ser-Tyr-Ser-Thr-Met(O)-Ser-Ile-Thr-Asp(OBzl)-Cys(MBzl)-Arg(MBS)-Glu(OBzl)-Thr-Gly-Ser-Ser-Lys(Z)-Tyr-Pro-Asn-Cys(MBzl)-Ala-Tyr-Lys(Z)-Thr-Thr-Gln-Ala-Asn-Lys(Z)-His-Ile-Ile-Val-Ala-Cys(MBzl)-Glu(OBzl)-Gly-Asn-Pro-Tyr-Val-Pro-Val-His-Phe-Asp(OBzl)-Ala-Ser-Val-OBzl, [Abbreviated as Z(OMe)-(RNase 65-124)-OBzl or Z(OMe)-Cys(MBzl)-Lys(Z)-Asn-Gly-(69-124)-OBzl]. ————— Z(OMe)-(RNase 69-124)-OBzl (9.49 g, 1.2 mmol) was treated with TFA-anisole (50 ml- 10 ml) as usual, then dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets in vacuo for 3 hr and dissolved in DMSO-DMF-NMP (1:1:1, 70 ml) containing Et_3N (0.50 ml, 3.6 mmol). To this ice-chilled solution, the azide (prepared from 3.07 g, 3.6 mmol of Z(OMe)-Cys(MBzl)-Lys(Z)-Asn-Gly-NHNH₂) in DMF (20 ml) and Et_3N (0.50 ml, 3.6 mmol) were added. After stirring for 48 hr, the solution was concentrated and the residue was purified by procedure B followed by precipitation (6 times) from DMSO with MeOH to yield the protected hexacontapeptide ester (9.04 g, 88%), m.p. 246° dec., $[\alpha]_D^{23} - 22.5^\circ$ (c 1.0 in DMSO), $Rf_3 = 0$ (Found: C, 50.78; H, 6.21; N, 12.17. $C_{406}H_{543}N_{79}O_{114}S_{7.54} \cdot H_2O$ requires C, 51.05; H, 6.87; N, 11.59%).

Z(OMe)-(RNase 62-124)-OBzl [Z(OMe)-Asn-Val-Ala-(65-124)-OBzl]. ————— Z(OMe)-(RNase 65-124)-OBzl (9.03 g, 1.05 mmol) was treated with TFA-anisole (50 ml-10 ml) as usual and the N^α-deprotected peptide, isolated as mentioned above, was dissolved in DMSO-DMF-NMP (1:1:1, 70 ml) containing Et_3N (0.44 ml, 3.16 mmol). To this ice-chilled solution, the azide (prepared from 0.59 g, 3.16 mmol of Z(OMe)-Asn-Val-Ala-NHNH₂) in DMF (5 ml) and Et_3N (0.44 ml, 3.16 mmol) were added and the solution, after stirring for 48 hr, was concentrated. The residue was purified by procedure B followed

by precipitation (8 times) from DMSO with MeOH to yield the protected trihexacontapeptide ester (8.18 g, 88%), m.p. 239° dec., $[\alpha]_D^{23}$ -28.2° (c 0.9 in DMSO), $Rf_3 = 0$ (Found: C, 54.25; H, 6.38; N, 12.99. $C_{418}H_{563}N_{83}O_{118}S_{7.18}H_2O$ requires C, 54.64; H, 6.57; N, 12.66%).

Z(OMe)-(RNase 57-124)-OBzl, [Z(OMe)-Val-Cys(MBzl)-Ser-Gln-Lys(Z)-(62-124)-OBzl]. ————— Z(OMe)-(RNase 62-124)-OBzl (8.17 g, 0.92 mmol) was treated with TFA-anisole (50 ml-10 ml) as usual and the N^α -deprotected peptide, isolated as mentioned above, was dissolved in DMSO-DMF-HMPA (1:1:1, 60 ml) containing Et_3N (0.38 ml, 2.77 mmol). To this ice-chilled solution, the azide (prepared from 3.22 g, 3.23 mmol of Z(OMe)-Val-Cys(MBzl)-Ser-Gln-Lys(Z)-NHNH₂) in DMF (20 ml) and Et_3N (0.45 ml, 3.23 mmol) were added and the solution, after stirring for 48 hr, was concentrated. The residue was purified by procedure B followed by precipitation (6 times) from DMSO with MeOH to yield the protected octahexacontapeptide ester (8.00 g, 90%), m.p. 240° dec., $[\alpha]_D^{23}$ -26.5° (c 1.1 in DMSO), $Rf_3 = 0$ (Found: C, 52.40; H, 6.38; N, 12.59. $C_{456}H_{616}N_{90}O_{128}S_{8.40}H_2O$ requires C, 52.74; H, 6.76; N, 12.14%).

Z(OMe)-(RNase 53-124)-OBzl, [Z(OMe)-Asp(OBzl)-Val-Gln-Ala-(57-124)-OBzl]. ————— Z(OMe)-(RNase 57-124)-OBzl (7.99 g, 0.83 mmol) was treated with TFA-anisole (40 ml-8 ml) for 80 min and the N^α -deprotected peptide, isolated as mentioned above, was dissolved in DMSO-DMF-HMPA (1:1:1, 70 ml) containing Et_3N (0.34 ml, 2.48 mmol). To this ice-chilled solution, the azide (prepared from 2.03 g, 2.90 mmol of Z(OMe)-Asp(OBzl)-Val-Gln-Ala-NHNH₂) in DMF (10 ml) and Et_3N (0.40 ml, 2.90 mmol) were added and the solution, after stirring for 96 hr, was concentrated. The residue was purified by procedure B followed by precipitation (4 times) from DMSO with MeOH to yield the protected doheptacontapeptide ester (7.59 g,

90%), m.p. 244° dec., $[\alpha]_D^{23} -27.4^\circ$ (c 0.7 in DMSO), (Found: C, 53.14; H, 6.31; N, 12.88. $C_{480}H_{649}N_{95}O_{135}S_{8.34}H_2O$ requires C, 53.48; H, 6.71; N, 12.35%).

Z(OMe)-(RNase 49-124)-OBzl, [Z(OMe)-Glu(OBzl)-Ser-Leu-Ala-(53-124)-OBzl]. ————— Z(OMe)-(RNase 53-124)-OBzl (7.85 g, 0.75 mmol) was treated with TFA-anisole (40 ml-8 ml) as usual and the N^α -deprotected peptide, isolated as mentioned above, was dissolved in DMSO-DMF-HMPA (1:1:1, 80 ml) containing Et_3N (0.31 ml, 2.24 mmol). To this ice-chilled solution, the azide (prepared from 1.79 g, 2.61 mmol of Z(OMe)-Glu(OBzl)-Ser-Leu-Ala-NHNH₂) in DMF (15 ml) and Et_3N (0.36 ml, 2.61 mmol) were added. After stirring for 48 hr, the solution was concentrated and the residue was purified by procedure B followed by precipitation (4 times) from DMSO with MeOH to yield the protected hexaheptacontapeptide ester (7.40 g, 93%), m.p. 244° dec., $[\alpha]_D^{23} -28.3^\circ$ (c 0.7 in DMSO), (Found: C, 53.81; H, 6.44; N, 12.85. $C_{504}H_{683}N_{99}O_{142}S_{8.30}H_2O$ requires C, 54.06; H, 6.69; N, 12.39%).

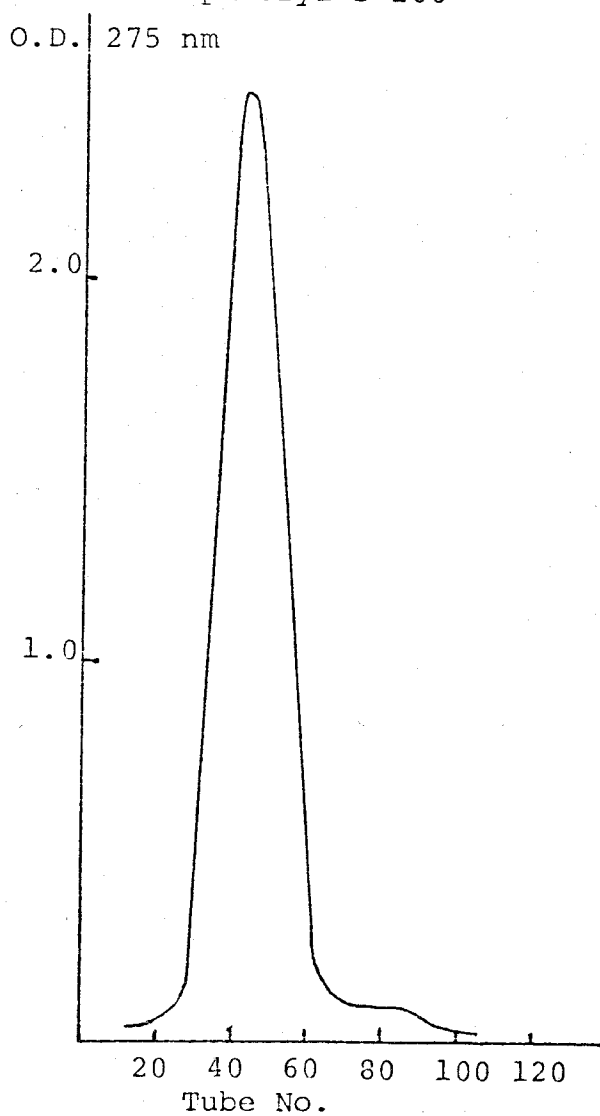
Z(OMe)-(RNase 46-124)-OBzl, [Z(OMe)-Phe-Val-His-(49-124)-OBzl]. ————— Z(OMe)-(RNase 49-124)-OBzl (7.39 g, 0.69 mmol) was treated with TFA-anisole (40 ml-8 ml) as usual and the N^α -deprotected peptide, isolated as mentioned above, was dissolved in DMSO-DMF-HMPA (1:1:1, 70 ml) containing Et_3N (0.29 ml, 2.08 mmol). To this ice-chilled solution, the azide (prepared from 1.41 g, 2.43 mmol of Z(OMe)-Phe-Val-His-NHNH₂) in DMF (9 ml) and N-methylmorpholine (0.27 ml, 2.43 mmol) were added and the solution was stirred for 48 hr. Additional azide (prepared from 0.81 g, 1.39 mmol of the hydrazide) in DMF (5 ml) and N-methylmorpholine (1.5 ml, 1.39 mmol) were added. Stirring was continued for 48 hr, until the solution became ninhydrin negative. The solvent was removed by evaporation and the residue was purified by procedure B fol-

lowed by precipitation (6 times) from DMSO with MeOH to yield the protected nonaheptacontapeptide ester (7.26 g, 95%), m.p. 240° dec., $[\alpha]_D^{23}$ -25.5° (c 0.5 in DMSO), (Found: C, 54.53; H, 6.36; N, 13.22. $C_{524}H_{708}N_{104}O_{145}S_{8.25}$ H_2O requires C, 54.77; H, 6.65; N, 12.68%).

Z(OMe)-(RNase 41-124)-OBzl, [Z(OMe)-Lys(Z)-Pro-Val-Asn-Thr-(46-124)-OBzl]. ————— Z(OMe)-(RNase 46-124)-OBzl (6.24 g, 0.57 mmol) was treated with TFA-anisole (35 ml-7 ml) and the N^α-deprotected peptide, isolated as mentioned above, was dissolved in DMSO-DMF-HMPA (1:1:1, 50 ml) containing Et₃N (0.32 ml, 2.26 mmol). To this ice-chilled solution, the azide (prepared from 1.72 g, 1.98 mmol of Z(OMe)-Lys(Z)-Pro-Val-Asn-Thr-NHNH₂) in DMF-DMSO (1:1, 12 ml) and Et₃N (0.28 ml, 1.98 mmol) were added and the solution was stirred for 48 hr. Additional azide (prepared from 0.98 g, 1.13 mmol of the hydrazide) in DMF-DMSO (1:1, 8 ml) and Et₃N (0.16 ml, 1.13 mmol) were added and the solution, after further stirring for 48 hr, was concentrated. The residue was purified by procedure B followed by precipitation (2 times) from DMSO with DMF, then DMSO with MeOH to yield the protected tetraoctacontapeptide ester (5.69 g, 86%), m.p. 249° dec., $[\alpha]_D^{23}$ - 28.3° (c 0.4 in DMSO), (Found: C, 53.42; H, 6.42; N, 13.20. $C_{556}H_{755}N_{111}O_{154}S_{8.39}$ H_2O requires C, 53.77; H, 6.76; N, 12.53%).

For testing purity, the product (250 mg), dissolved in 5% H₂O-DMSO (4 ml), was applied to a column of Sephacryl S-200 (2.5 x 131 cm), which was eluted with 5% H₂O-DMSO at a flow rate of 39 ml/hr. Individual fractions (7 ml each) were collected and absorption at 275 nm was measured. A single peak with small tailing was detected (Fig. 3). The desired fractions (Tube Nos. 32-60) were collected and the solvent was removed by evaporation and the residue was treated with MeOH to give a powder (233 mg, 93%). No significant difference was observed in amino acid analyses, before (Table 4) and after the gel-filtration: Asp 10.17, Thr 6.52, Ser 6.39, Glu

Fig.3. Gel-filtration of
Z(OMe)-(RNase 41-124)-OBzl
on Sephacryl S-200



8.07, Pro 3.96, Gly 3.09, Ala 7.03, Val 8.79, Met + Met(O) 0.82, Ile 2.37, Leu 0.97, Tyr 5.26, Phe 2.00, Lys 6.18, His 2.69, Arg 1.02 (average recovery 91%).

Section 5. Synthesis of the Protected S-Protein (Positions 21-124) and the Protected S-Peptide (Positions 1-20)

Z(OMe)-Cys(MBzl)-NHNH-Troc. ————— DCC (11.30 g, 55 mmol) was added to a stirred solution of Z(OMe)-Cys(MBzl)-OH (20.27 g, 50 mmol) and Troc-NHNH₂ (11.40 g, 55 mmol) in AcOEt (250 ml). After 48 hr, the solution was filtered, the filtrate washed with 10% citric acid, 5% NaHCO₃ and H₂O, dried over Na₂SO₄ and concentrated. Trituration with ether followed by recrystallization from AcOEt and ether afforded the substituted hydrazide (20.20 g, 68%), m.p. 126-129°, $[\alpha]_D^{27}$ -21.8° (c 0.9 in DMF), R_f_2 0.51 (Found: C, 46.58; H, 4.45; N, 7.07. C₂₃H₂₆N₃O₇SCl₃ requires C, 46.43; H, 4.41; N, 7.06%).

Z(OMe)-Arg(MBS)-Cys(MBzl)-NHNH-Troc. ————— Z(OMe)-Cys(MBzl)-NHNH-Troc (8.15 g, 14 mmol) was treated with TFA-anisole (16 ml- 4 ml) as usual, then excess TFA was removed by evaporation. The oily residue was washed with n-hexane and dissolved in 4.2 N HCl-dioxane (4.0 ml, 16 mmol). The solvent was evaporated and the resulting oil, after washing with n-hexane, was dissolved in DMF (80 ml) together with Et₃N (1.9 ml, 14 mmol), HOBT (2.22 g, 16 mmol) and Z(OMe)-Arg(MBS)-OH (7.66 g, 15 mmol). After addition of DCC (3.39 g, 16 mmol), the solution was stirred for 48 hr, filtered and the filtrate was concentrated. The residue was purified by procedure A followed by recrystallization from AcOEt and ether to afford the protected dipeptide (11.33 g, 90%), m.p. 95-98°, $[\alpha]_D^{22}$ -15.6° (c 0.5 in MeOH), R_f_1 0.69 (Found: C, 47.08; H, 4.65; N, 10.65. C₃₆H₄₄N₇O₁₁S₂Cl₃ requires C, 46.93; H, 4.81; N, 10.64%).

Z(OMe)-Asp(OBzl)-Arg(MBS)-Cys(MBzl)-NHNH-Troc [23]. —————
The above protected dipeptide (11.33 g, 12 mmol) was treated with

TFA-anisole (23 ml-5.6 ml) as usual, then dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets in vacuo for 3 hr and dissolved in DMF (100 ml) together with Et_3N (3.4 ml, 25 mmol) and $\text{Z(OMe)-Asp(OBzl)-ONP}$ (7.51 g, 15 mmol). After stirring for 48 hr, the solution was concentrated and the residue was purified by procedure A followed by recrystallization from AcOEt and ether to give the protected tripeptide (10.50 g, 76%), m.p. 101-105°, $[\alpha]_D^{22}$ -11.5° (c 1.3 in DMF), Rf_1 0.66 (Found: C, 50.86; H, 4.97; N, 10.34. $\text{C}_{47}\text{H}_{55}\text{N}_8\text{O}_{14}\text{S}_2\text{Cl}_3$ requires C, 50.11; H, 4.92; N, 9.95%).

$\text{Z(OMe)-Asp(OBzl)-Arg(MBS)-Cys(MBzl)-NHNH}_2$, $\text{Z(OMe)-(RNase 38-40)-NHNH}_2$ ————— The above protected tripeptide (10.36 g, 9.2 mmol) in AcOH (100 ml) was treated with Zn powder (6.0 g, 10 equiv.) for 48 hr and the solvent was evaporated in vacuo at the bath temperature below 25°. The residue was treated with a saturated solution of EDTA to give a gelatinous mass, which was washed with 5% Na_2CO_3 and H_2O and recrystallized from dioxane and ether to afford the protected tripeptide hydrazide (7.01 g, 80%), m.p. 161-167°, $[\alpha]_D^{23}$ - 23.4° (c 1.4 in DMSO), Rf_1 0.53. Amino acid analysis: Asp 1.00, Arg 0.82, average recovery 87% (Found: C, 54.41; H, 5.59; N, 11.33. $\text{C}_{44}\text{H}_{54}\text{N}_8\text{O}_{12}\text{S}_2\cdot\text{H}_2\text{O}$ requires C, 54.53; H, 5.82; N, 11.56%).

$\text{Z(OMe)-Thr-Lys(Z)-OH}$. ————— Z(OMe)-Thr-OPCP (42.40 g, 80 mmol) was added to a stirred solution of Lys(Z) (22.40 g, 80 mmol) in 50% aqueous pyridine (220 ml) containing Et_3N (21.6 ml, 0.16 mol). After 48 hr, the solvent was evaporated and the residue was purified by procedure C followed by recrystallization from MeOH and AcOEt to yield the protected dipeptide (27.03 g, 62%), m.p. 141-145°, $[\alpha]_D^{22}$ + 13.0° (c 0.5 in DMF), Rf_1 0.35 (Found: C, 59.70; H, 6.44; N, 7.80. $\text{C}_{27}\text{H}_{35}\text{N}_3\text{O}_9$ requires C, 59.43; H, 6.46; N, 7.70%).

Z(OMe)-Leu-Thr-Lys(Z)-OH. ————— Z(OMe)-Thr-Lys(Z)-OH
 (12.40 g, 23 mmol) was treated with TFA-anisole (20 ml-6 ml) as usual, then dry ether was added. The resulting powder isolated as mentioned above, was dissolved in DMF (80 ml) together with Et₃N (9.5 ml, 69 mmol) and Z(OMe)-Leu-ONP (9.61 g, 23 mmol). After stirring for 48 hr, the solution was concentrated and the residue was purified by procedure C followed by recrystallization from AcOEt to give the protected tripeptide (12.42 g, 83%), m.p. 99-104°, $[\alpha]_D^{23} + 0.6$ (c 0.8 in DMF), Rf_1 0.57 (Found: C, 59.89; H, 7.00; N, 8.71. C₃₃H₄₆N₄O₁₀ requires C, 60.17; H, 7.04; N, 8.51%).

Z(OMe)-Asn-Leu-Thr-Lys(Z)-OH. ————— Z(OMe)-Leu-Thr-Lys(Z)-OH (9.01 g, 13.7 mmol) was treated with TFA-anisole (9 ml-4.5 ml) as usual, then dry ether was added. The resulting powder, isolated as mentioned above, was dissolved in DMF (90 ml) together with Et₃N (5.7 ml, 41 mmol) and Z(OMe)-Asn-ONP (7.40 g, 18 mmol). After stirring for 48 hr, the solution was concentrated and the residue was treated with 5% citric acid and ether to form a powder, which was washed with 5% citric acid and H₂O and precipitated from DMF with AcOEt to afford the protected tetrapeptide (8.13 g, 77%), m.p. 178-181°, $[\alpha]_D^{22} -14.3^\circ$ (c 1.4 in DMF), Rf_1 0.32 (Found: C, 57.44; H, 6.80; N, 10.82. C₃₇H₅₂N₆O₁₂ requires C, 57.50; H, 6.78; N, 10.88%).

Z(OMe)-Arg(MBS)-Asn-Leu-Thr-Lys(Z)-OH. ————— DCC (2.47 g, 12 mmol) was added to a stirred solution of Z(OMe)-Arg(MBS)-OH (6.15 g, 12 mmol) and DNP-OH (2.23 g, 12 mmol) in AcOEt-DMF (30 ml-5 ml). After 5 hr, the solution was filtered and the filtrate was submitted to the following condensation reaction. Z(OMe)-Asn-Leu-Thr-Lys(Z)-OH (8.50 g, 11 mmol) was treated with TFA-anisole (16.8 ml-4.2 ml) and the N^α-deprotected peptide, isolated as mentioned above, was dissolved in DMF (50 ml) containing Et₃N (4.6 ml, 33

mmol). This solution was mixed with the solution containing the DNP ester. After stirring for 48 hr, the solution was concentrated and the residue was purified by washing with 5% citric acid and H₂O followed by recrystallization from MeOH and AcOEt to afford the protected pentapeptide (6.80 g, 56%), m.p. 168-171°, $[\alpha]_D^{23}$ - 6.8° (c 0.1 in DMF), Rf₂ 0.14 (Found: C, 54.59; H, 6.62; N, 12.63. C₅₀H₇₀N₁₀O₁₆S requires C, 54.63; H, 6.42; N, 12.74%).

Z(OMe)-Arg(MBS)-Asn-Leu-Thr-Lys(Z)-OMe. ————— An ethereal solution of diazomethane was added to an ice-chilled solution of Z(OMe)-Arg(MBS)-Asn-Leu-Thr-Lys(Z)-OH (6.34 g, 5.8 mmol) in DMF-MeOH (1:1, 100 ml) until the yellow color persisted for 30 min. After addition of a few drops of AcOH, the solution was concentrated and the residue was triturated with ether. The resulting powder was washed with 5% Na₂CO₃ and H₂O and precipitated from DMF with AcOEt to give the protected pentapeptide ester (4.48 g, 70%), m.p. 158-160°, $[\alpha]_D^{22}$ - 12.1° (c 1.0 in DMF), Rf₁ 0.61 (Found: C, 55.01; H, 6.38; N, 12.55. C₅₁H₇₂N₁₀O₁₆S requires C, 55.02; H, 6.52; N, 12.58%).

Z(OMe)-Arg(MBS)-Asn-Leu-Thr-Lys(Z)-NHNH₂, Z(OMe)-(RNase 33-37)-NHNH₂ [24]. ————— The above methyl ester (4.01 g, 3.6 mmol) in DMF (40 ml) was treated with 80% hydrazine hydrate (2.3 ml, 10 equiv.) overnight. The solvent was evaporated and the residue was triturated with MeOH. Precipitation of the resulting mass from DMF with MeOH afforded the hydrazide (3.30 g, 82%), m.p. 237-240°, $[\alpha]_D^{22}$ - 11.6° (c 1.0 in DMSO), Rf₁ 0.55. Amino acid analysis: Asp 1.01, Thr 0.94, Leu 1.00, Lys 1.13, Arg 0.87, average recovery 94% (Found: C, 53.76; H, 6.30; N, 14.88. C₅₀H₇₂N₁₂O₁₅S requires C, 53.94; H, 6.52; N, 15.10%).

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Z(OMe)-Met-Lys(Z)-Ser-OMe. ————— Z(OMe)-Lys(Z)-Ser-OMe

(16.37 g, 30 mmol) was treated with TFA-anisole (33 ml-8.2 ml) as usual and dry ether was added. The resulting powder was dissolved in 4.21 N HCl-dioxane (7.13 ml, 30 mmol), the solvent was evaporated and the residue was treated with ether. The resulting powder was collected by filtration, dried over KOH pellets in vacuo for 3 hr and dissolved in DMF (150 ml) together with Et₃N (4.14 ml, 30 mmol) and Z(OMe)-Met-OH (9.40 g, 30 mmol). After addition of DCC (6.80 g, 33 mmol), the solution was stirred for 48 hr, filtered and the filtrate was concentrated. The residue was purified by procedure B followed by precipitation from DMF with AcOEt to afford the protected tripeptide ester (14.70 g, 72%), m.p. 188-190°, $[\alpha]_D^{23}$ - 12.9° (c 0.2 in DMF), Rf₁ 0.60 (Found: C, 56.87; H, 6.62; N, 8.42. C₃₂H₄₄N₄O₁₀ requires C, 56.79; H, 6.55; N, 8.28%).

Z(OMe)-Met(O)-Lys(Z)-Ser-OMe. ————— NaIO₄ (2.35 g, 11 mmol) in H₂O (50 ml) was added to an ice-chilled solution of Z(OMe)-Met-Lys(Z)-Ser-OMe (6.77 g, 10 mmol) in DMF-MeOH (30 ml-50 ml). After stirring at 4° for 24 hr, the solution was concentrated and the residue was treated with ether and H₂O. The resulting powder was precipitated from DMF with MeOH to afford the sulphoxide (6.20 g, 90%), m.p. 207-211°, $[\alpha]_D^{22}$ + 2.1° (c 0.9 in DMF), Rf₁ 0.79, Rf₂ 0.62 (Found: C, 55.29; H, 6.41; N, 8.06. C₃₂H₄₄N₄O₁₁S requires C, 55.48; H, 6.40; N, 8.09%).

Z(OMe)-Met(O)-Lys(Z)-Ser-NHNH₂, Z(OMe)-(RNase 30-32)-NHNH₂
[25]. ————— Z(OMe)-Met(O)-Lys(Z)-Ser-OMe (6.03 g, 8.7 mmol) in DMF-MeOH (40 ml-10 ml) was treated with 80% hydrazine hydrate (4.4 ml, 8 equiv.) overnight. The solvent was evaporated off and the residue was precipitated from DMF with MeOH to give the hydrazide (4.97 g, 82%), m.p. 221-224°, $[\alpha]_D^{22}$ + 2.0° (c 1.0 in DMSO), Rf₁ 0.61, Rf₂ 0.20. Amino acid analysis: Met + Met(O) 0.82, Lys 1.00, Ser 0.71, average recovery 90% (Found: C, 53.54; H, 6.67;

N, 12.10. $C_{31}H_{44}N_6O_{10}$ requires C, 53.74; H, 6.40; N, 12.13%).

Z(OMe)-Gln-Met-OMe. ————— Z(OMe)-Gln-ONP (64.65 g, 0.15 mol) and Et_3N (20.7 ml, 0.15 mol) were added to a stirred solution of H-Met-OMe (prepared from 29.85 g, 0.15 mol of the hydrochloride with 20.7 ml, 0.15 mol of Et_3N) in DMF (300 ml). After 48 hr, the solution was concentrated and the residue was purified by procedure B followed by recrystallization from dioxane and MeOH to give the protected dipeptide ester (46.41 g, 68%), m.p. 162-170°, $[\alpha]_D^{23}$ - 7.0° (c 1.4 in DMF), Rf_1 0.43 (Found: C, 53.12; H, 6.63; N, 9.33. $C_{20}H_{29}N_3O_7S$ requires C, 52.73; H, 6.42; N, 9.23%).

Z(OMe)-Gln-Met(O)-OMe. ————— $NaIO_4$ (1.93 g, 9 mmol) in H_2O (20 ml) was added to an ice-chilled solution of Z(OMe)-Gln-Met-OMe (3.65 g, 8 mmol) in MeOH-DMF (40 ml-25 ml). After stirring at 4° for 12 hr, the solution was concentrated and the residue was washed with H_2O to remove the inorganic salt; some of the product was lost, due of its water solubility. Precipitation from DMF with ether afforded the sulphoxide (1.60 g, 40%), m.p. 185-186°, $[\alpha]_D^{23}$ - 15.9° (c 1.8 in DMF), Rf_1 0.70, Rf_2 0.55 (Found: C, 50.93; H, 6.13; N, 8.91. $C_{20}H_{29}N_3O_8S$ requires C, 50.94; H, 6.20; N, 8.91%).

Z(OMe)-Asn-Gln-Met(O)-OMe. ————— Z(OMe)-Gln-Met(O)-OMe (7.77 g, 16.5 mmol) was treated with TFA-anisole (30 ml-8 ml), as usual, then dry ether was added. The resulting powder, isolated as mentioned above, was dissolved in DMF-DMSO (100 ml-50 ml) together with Et_3N (4.55 ml, 33 mmol), HOBT (0.50 g, 3.3 mmol) and Z(OMe)-Asn-ONP (6.89 g, 16.5 mmol). After stirring for 24 hr, the solution was concentrated and the residue was purified by washing with 5% citric acid and H_2O followed by precipitations (2 times) from DMSO with AcOEt to yield the protected tripeptide ester (7.01 g, 73%), m.p. 216-220°, $[\alpha]_D^{23}$ - 19.6° (c 0.5 in DMSO), Rf_1 0.37 (Found: C,

47.18; H, 6.19; N, 11.41. $C_{24}H_{35}N_5O_{10}S \cdot 1.5 H_2O$ requires C, 47.05; H, 6.25; N, 11.43%). Attempts to wash with 5% Na_2CO_3 or precipitate from DMSO with MeOH or EtOH produced a pasty material that was difficult to collect by filtration.

Z(OMe)-Asn-Gln-Met(O)-NHNH₂, Z(OMe)-(RNase 27-29)-NHNH₂ [26].
 ————— Z(OMe)-Asn-Gln-Met(O)-OMe (4.82 g, 8.2 mmol) in DMSO-MeOH (20 ml-5 ml) was treated with 80% hydrazine hydrate (2.06 ml, 4 equiv.) at 4° overnight. The solvent was evaporated and the residue was precipitated twice from DMSO with MeOH to afford the hydrazide (4.68 g, 97%), m.p. 226-229°, $[\alpha]_D^{20} - 23.5^\circ$ (c 0.9 in DMSO), Rf_1 0.14. Amino acid analysis: Asp 1.00, Glu 1.03, Met + Met(O) 0.71, average recovery 98% (Found: C, 46.53; H, 6.10; N, 16.13. $C_{23}H_{35}N_7O_9S \cdot 1/2 H_2O$ requires C, 46.45; H, 6.10; N, 16.49%).

Z(OMe)-Asn-Tyr-OMe. ————— Z(OMe)-Asn-ONP (12.98 g, 31.1 mmol) and Et₃N (4.29 ml, 31.1 mmol) were added to a stirred solution of H-Tyr-OMe (prepared from 7.29 g, 37.3 mmol of the hydrochloride with 5.15 ml, 37.3 mmol of Et₃N) in DMF (100 ml). After 24 hr, the solution was concentrated and the residue was purified by procedure B followed by precipitation (2 times) from MeOH and ether to give the protected dipeptide ester (10.61 g, 72%), m.p. 171-172°, $[\alpha]_D^{20} + 8.2^\circ$ (c 1.1 in DMF), Rf_1 0.45 (Found: C, 58.37; H, 5.83; N, 8.87. $C_{23}H_{27}N_3O_8$ requires C, 58.34; H, 5.75; N, 8.88%).

Z(OMe)-Asn-Tyr-NHNH₂. ————— Z(OMe)-Asn-Tyr-OMe (6.05 g, 12.8 mmol) in DMF-MeOH (30 ml-5 ml) was treated with 80% hydrazine hydrate (6.4 ml, 8 equiv.) overnight and MeOH (200 ml) was added. The resulting gelatinous mass was precipitated twice from DMF with MeOH to afford the hydrazide (5.63 g, 93%), m.p. 215-218°, $[\alpha]_D^{23} - 1.7^\circ$ (c 0.6 in DMSO), Rf_1 0.22 (Found: C, 55.99; H, 5.78 N, 14.95. $C_{22}H_{27}N_5O_7$ requires C, 55.80; H, 5.75; N, 14.79%).

Z(OMe)-Asn-Tyr-Cys(MBzl)-OMe. ————— The azide (prepared from 5.02 g, 10.6 mmol of Z(OMe)-Asn-Tyr-NHNH₂) in DMF (20 ml) and Et₃N (1.61 ml, 11.7 mmol) were added to an ice-chilled solution of H-Cys(MBzl)-OMe (prepared from 6.17 g, 14.7 mmol of the Z(OMe)-derivative by the usual TFA treatment followed by neutralization with 2.23 ml, 16.2 mmol of Et₃N) in DMF (45 ml). After stirring for 24 hr, the solution was concentrated and the residue was purified by procedure B followed by precipitation twice from DMF with MeOH to yield the methyl ester (6.02 g, 82%), m.p. 198-200°, $[\alpha]_D^{23}$ -37.3° (c 0.7 in DMF), R_f₁ 0.68 (Found: C, 58.53; H, 5.65; N, 8.20. C₃₄H₄₀N₄O₁₀S requires C, 58.61; H, 5.79; N, 8.04%).

Z(OMe)-Asn-Tyr-Cys(MBzl)-NHNH₂, Z(OMe)-(RNase 24-26)-NHNH₂
 [27]. ————— Z(OMe)-Asn-Tyr-Cys(MBzl)-OMe (6.02 g, 8.7 mmol) in DMF-MeOH (30 ml-5 ml) was treated with 80% hydrazine hydrate (4.3 ml, 10 equiv.) overnight. Addition of MeOH (200 ml) gave a gelatinous mass, which was precipitated from DMF with MeOH to give the hydrazide (5.33 g, 88%), m.p. 224-226°, $[\alpha]_D^{20}$ -20.3° (c 1.2 in DMSO), R_f₁ 0.39. Amino acid analysis: Asp 1.00, Tyr 1.01, average recovery 94% (Found: C, 56.62; H, 5.79; N, 11.97. C₃₈H₄₀N₆O₉S requires C, 56.88; H, 5.79; N, 12.06%).

Z(OMe)-Ser-Ser-OMe. ————— The azide (prepared from 10.05 g, 36 mmol of Z(OMe)-Ser-NHNH₂) in DMF (70 ml) and Et₃N (4.90 ml, 36 mmol) were added to an ice-chilled solution of H-Ser-OMe (prepared from 7.18 g, 46 mmol of the hydrochloride with 6.36 ml, 46 mmol of Et₃N) in DMF (35 ml). After stirring for 24 hr, the solution was concentrated. Trituration with EtOH followed by precipitation from DMF with EtOH afforded the protected dipeptide ester (10.22 g, 60%), m.p. 124-127°, $[\alpha]_D^{23}$ +12.1° (c 0.4 in DMF), R_f₁ 0.52 (Found: C, 51.47; H, 5.91; N, 7.45. C₁₆H₂₂N₂O₈ requires C, 51.89; H, 5.99; N, 7.56%).

Z(OMe)-Ser-Ser-Ser-OMe. ————— Z(OMe)-Ser-Ser-OMe (8.10 g, 22 mmol) was treated with TFA-anisole (24 ml-8 ml), as usual, and dry ether was added. The resulting powder, isolated as mentioned above, was dissolved in DMF (80 ml) containing Et₃N (3.02 ml, 22 mmol). To this ice-chilled solution, the azide (prepared from 6.20 g, 22 mmol of Z(OMe)-Ser-NHNH₂) in DMF (50 ml) and Et₃N (3.02 ml, 22 mmol) were added. After stirring for 24 hr, the solution was concentrated. Trituration with EtOH followed by precipitation from DMF with EtOH afforded the protected tripeptide ester (7.62 g, 76%), m.p. 188-189°, $[\alpha]_D^{23} + 7.9^\circ$ (c 0.5 in DMF), Rf_1 0.67 (Found: 50.18; H, 5.95; N, 8.90. C₁₉H₂₇N₃O₁₀ requires C, 49.89; H, 5.95; N, 9.19%).

Z(OMe)-Ser-Ser-Ser-NHNH₂, Z(OMe)-(RNase 21-23)-NHNH₂ [28].
 ————— Z(OMe)-Ser-Ser-Ser-OMe (5.01 g, 11 mmol) in DMF-MeOH (20 ml-30 ml) was treated with 80% hydrazine hydrate (2.74 ml, 4 equiv.) overnight. The solvent was removed by evaporation and the residue was precipitated from DMF with MeOH to give the hydrazide (4.42 g, 88%), m.p. 209-214°, $[\alpha]_D^{20} + 5.2^\circ$ (c 0.8 in DMSO), Rf_1 0.41 (Found: C, 46.64; H, 5.94; N, 15.06. C₁₈H₂₇N₅O₉ · 1/2 H₂O requires C, 46.35; H, 6.05; N, 15.02%).

Z(OMe)-Asp(OBzl)-Arg(MBS)-Cys(MBzl)-Lys(Z)-Pro-Val-Asn-Thr-Phe-Val-His-Glu(OBzl)-Ser-Leu-Ala-Asp(OBzl)-Val-Gln-Ala-Val-Cys(MBzl)-Ser-Gln-Lys(Z)-Asn-Val-Ala-Cys(MBzl)-Lys(Z)-Asn-Gly-Gln-Thr-Asn-Cys(MBzl)-Tyr-Gln-Ser-Tyr-Ser-Thr-Met(O)-Ser-Ile-Thr-Asp(OBzl)-Cys(MBzl)-Arg(MBS)-Glu(OBzl)-Thr-Gly-Ser-Ser-Lys(Z)-Tyr-Pro-Asn-Cys(MBzl)-Ala-Tyr-Lys(Z)-Thr-Thr-Gln-Ala-Asn-Lys(Z)-His-Ile-Ile-Val-Ala-Cys(MBzl)-Glu(OBzl)-Gly-Asn-Pro-Tyr-Val-Pro-Val-His-Phe-Asp(OBzl)-Ala-Ser-Val-OBzl, [abbreviated as Z(OMe)-(RNase 38-124)-OBzl or Z(OMe)-Asp(OBzl)-Arg(MBS)-Cys(MBzl)-(41-124)-OBzl]. ————— Z(OMe)-(RNase 41-124)-OBzl (6.21 g, 0.53

mmol) was treated with TFA-anisole (28 ml-7 ml), then dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets in vacuo for 3 hr and dissolved in DMSO-DMF-HMPA (1:1:1, 50 ml) containing Et₃N (0.29 ml, 2.12 mmol). To this ice-chilled solution, the azide (prepared from 1.51 g, 1.59 mmol of Z(OMe)-Asp(OBzl)-Arg(MBS)-Cys(MBzl)-NHNH₂) in DMF (12 ml) and Et₃N (0.22 ml, 1.59 mmol) were added and the mixture was stirred at 4° for 72 hr. The additional azide (prepared from 1.01 g, 1.06 mmol of the hydrazide) in DMF (5 ml) and Et₃N (0.15 ml, 1.06 mmol) were added and the mixture, after stirring for additional 48 hr, was concentrated. The residue was purified by procedure B followed by precipitation (4 times) from DMSO with MeOH to yield the protected hepta-octacontapeptide ester (5.62 g, 85%), m.p. 246° dec., $[\alpha]_D^{23}$ -29.4° (c 0.4 in DMSO), R_f = 0 (Found: C, 53.62; H, 6.31; N, 12.41. C₅₉₁H₇₉₇N₁₁₇O₁₆₃S₁₀ · 38 H₂O requires C, 53.96; H, 6.69; N, 12.46%).

Z(OMe)-(RNase 33-124)-OBzl [Z(OMe)-Arg(MBS)-Asn-Leu-Thr-Lys(Z)-(38-124)-OBzl]. ————— Z(OMe)-(RNase 38-124)-OBzl (5.61 g, 0.45 mmol) was treated with TFA-anisole (24 ml-6 ml) and the N^α-deprotected peptide, isolated as mentioned above, was dissolved in DMSO-DMF-HMPA (1:1:1, 40 ml) containing Et₃N (0.25 ml, 1.8 mmol). To this ice-chilled solution, the azide (prepared from 1.50 g, 1.35 mmol of Z(OMe)-Arg(MBS)-Asn-Leu-Thr-Lys(Z)-NHNH₂) in DMF (8 ml) and Et₃N (0.19 ml, 1.35 mmol) were added and the mixture was stirred for 72 hr. The additional azide (prepared from 1.00 g, 0.90 mmol of the hydrazide) in DMF (6 ml) and Et₃N (0.12 ml, 0.90 mmol) were added and the mixture, after stirring for additional 72 hr, was concentrated. The product was purified by procedure B followed by precipitation (2 times) from DMSO with DMF. Further precipitations from DMSO with n-BuOH and from DMSO with MeOH afforded the protected dononacontapeptide ester, free from contamination of the acyl component on tlc (4.96 g, 82%),

m.p. 251° dec., $[\alpha]_D^{23} - 31.9^\circ$ (c 0.4 in DMSO), $R_f = 0$ (Found: C, 52.45; H, 6.25; N, 12.12. $C_{632}H_{857}N_{127}O_{175}S_{11} \cdot 50 H_2O$ requires C, 53.13; H, 6.75; N, 12.45%).

Z(OMe)-(RNase 30-124)-OBzl [Z(OMe)-Met(O)-Lys(Z)-Ser-(33-124)-OBzl]. ————— Z(OMe)-(RNase 33-124)-OBzl (4.95 g, 0.37 mmol) was treated with TFA-anisole (24 ml-6 ml) and the N^α -deprotected peptide, isolated as mentioned above, was dissolved in DMSO-DMF-HMPA (1:1:1, 40 ml) containing Et_3N (0.21 ml, 1.52 mmol). To this ice-chilled solution, the azide (prepared from 0.77 g, 1.11 mmol of Z(OMe)-Met(O)-Lys(Z)-Ser-NHNH₂) in DMF (6 ml) and Et_3N (0.16 ml, 1.16 mmol) were added and the mixture was stirred for 72 hr. The additional azide (prepared from 0.51 g, 0.74 mmol of the hydrazide) in DMF (5 ml) and Et_3N (0.10 ml, 0.74 mmol) were added and stirring was continued for 48 hr. The third batch of azide (prepared from 0.51 g, 0.74 mmol of the hydrazide) in DMF (5 ml) and Et_3N (0.10 ml, 0.74 mmol) were added and the reaction was continued for an additional 48 hr, until the solution became ninhydrin negative. The solvent was evaporated and the residue was purified by procedure B followed by precipitation (3 times) from DMSO with DMF, then once from DMSO with MeOH to afford the protected pentanonacontapeptide ester with no contamination of the acyl component on tlc (4.18 g, 81%), m.p. 248° dec., $[\alpha]_D^{23} - 24.2^\circ$ (c 0.4 in DMSO), $R_f = 0$ (Found: C, 53.57; H, 6.26; N, 12.48. $C_{632}H_{857}N_{127}O_{175}S_{12} \cdot 40 H_2O$ requires C, 53.79; H, 6.69; N, 12.56%).

Z(OMe)-(RNase 27-124)-OBzl [Z(OMe)-Asn-Gln-Met(O)-(30-124)-OBzl]. ————— Z(OMe)-(RNase 30-124)-OBzl (4.17 g, 0.30 mmol) was treated with TFA-anisole (25 ml-5 ml) and the N^α -deprotected peptide, isolated as mentioned above, was dissolved in DMSO-DMF-HMPA (1:1:1, 40 ml) containing Et_3N (0.17 ml, 1.23 mmol). To this ice-chilled solution, the azide (prepared from 0.88 g, 1.50

mmol of Z(OMe)-Asn-Gln-Met(O)-NHNH₂ in DMF-DMSO (1:1, 7 ml) and Et₃N (0.21 ml, 1.52 mmol) were added. After 72 hr, the 2nd batch of the azide (prepared from 0.35 g, 0.60 mmol of the hydrazide) in DMF-DMSO (1:1, 3 ml) and Et₃N (0.09 ml, 0.65 mmol) were added. After an additional 48 hr, the 3rd batch of the azide (prepared from 0.35 g, 0.60 mmol of the hydrazide) in DMSO-DMF (1:1, 3 ml) and Et₃N (0.09 ml, 0.65 mmol) were added and stirring was continued for a further 48 hr, until the solution became ninhydrin negative. The solvent was evaporated and the residue was purified by procedure B followed by precipitation from DMSO with DMF (2x). Further precipitations from DMSO with n-BuOH (2x) and from DMSO with MeOH (1x) afforded the protected octanonacontapeptide ester, free from contamination of the acyl component (3.47 g, 81%), m.p. 241° dec., $[\alpha]_D^{23} - 23.4^\circ$ (c 0.6 in DMSO), R_f = 0 (Found: C, 53.69; H, 6.43; N, 12.80. C₆₆₈H₉₁₂N₁₃₆O₁₈₈S₁₃·33 H₂O requires C, 53.97; H, 6.63; N, 12.81%).

Z(OMe)-(RNase 24-124)-OBzl [Z(OMe)-Asn-Tyr-Cys(MBzl)-(27-124)-OBzl]. ————— Z(OMe)-(RNase 27-124)-OBzl (3.46 g, 0.24 mmol) was treated with TFA-anisole (20 ml-4 ml) and the N^α-deprotected peptide, isolated as mentioned above, was dissolved in DMSO-DMF-HMPA (1:1:1, 30 ml) containing Et₃N (0.14 ml, 1.01 mmol). To this ice-chilled solution, the azide (prepared from 0.85 g, 1.22 mmol of Z(OMe)-Asn-Tyr-Cys(MBzl)-NHNH₂) in DMF (7 ml) and Et₃N (0.17 ml, 1.23 mmol) were added and, after 72 hr, the 2nd batch of the azide (prepared from 0.34 g, 0.49 mmol of the hydrazide) in DMF (3 ml) and Et₃N (67 μl, 0.49 mmol) were added. After an additional 48 hr, the same amounts of the azide and Et₃N were added and stirring was continued for 48 hr, until the solution became ninhydrin negative. The solution was concentrated and the residue was purified by procedure B followed by precipitation from DMSO with n-BuOH (3x). Further precipitation from DMSO with MeOH afforded the protected henhectapeptide ester, free from contamination.

tion by the acyl component on tlc (3.10 g, 86%), m.p. 240° dec., $[\alpha]_D^{23}$ -21.6° (c 0.6 in DMSO), R_f = 0 (Found: C, 54.12; H, 6.34; N, 12.60. $C_{692}H_{940}N_{140}O_{194}S_{14} \cdot 33 H_2O$ requires C, 54.08; H, 6.60; N, 12.76%).

Z(OMe)-(RNase 21-124)-OBzl [Z(OMe)-Ser-Ser-Ser-(24-124)-OBzl, Protected S-Protein]. ————— Z(OMe)-(RNase 24-124)-OBzl (3.09 g, 0.21 mmol) was treated with TFA-anisole (20 ml-4 ml) and the N-deprotected peptide, isolated as mentioned above, was dissolved in DMSO-DMF-HMPA (1:1:1, 25 ml) containing Et_3N (0.12 ml, 0.87 mmol). To this ice-chilled solution, the azide (prepared from 0.58 g, 1.26 mmol of Z(OMe)-Ser-Ser-Ser-NHNH₂) in DMF (5 ml) and Et_3N (0.18 ml, 1.30 mmol) were added and, after 72 hr's stirring, 3 batches of the azide (prepared from 0.19 g, 0.42 mmol of the hydrazide each) in DMF (3 ml) and Et_3N (58 μ l, 0.42 mmol each) were further added at 48 hr intervals. After addition of the last batch of the azide, stirring was further continued for 24 hr, until the solution became completely ninhydrin negative. H_2O (250 ml) was added and the resulting powder was purified by procedure B followed by precipitation from DMSO with n-BuOH (3x). Further precipitation from DMSO with MeOH afforded the protected tetrahepta peptide ester, free from contamination with the acyl component on tlc (2.59 g, 82%), m.p. 246°dec., $[\alpha]_D^{23}$ -21.1° (c 0.7 in DMSO), R_f = 0.

For testing purity, the sample (202 mg) was dissolved in 5% H_2O -DMSO (2 ml) and applied to a column of Sephacryl S-200 (3 x 142 cm), and eluted with 5% H_2O -DMSO. Individual fractions (10 ml each) were collected and absorption at 275 nm was determined. A single peak with a minor tailing was detected (Fig. 4). Fractions corresponding to the main peak (tube Nos. 22-55) were collected, the solvent was evaporated and the residue was treated with MeOH to give a powder (182 mg, 90%). No significant differences in amino acid analyses was noted, before (Table 5) and after gel-filtration:

Asp 14.21(14), Thr 7.49(8), Ser 9.44(12), Glu 9.09(9), Pro 3.81(4), Gly 3.06(3), Ala 6.71(7), Val 8.62(9), Met + Met(O) 2.17(3), Ile 2.09(3), Leu 2.05(2), Tyr 6.42(6), Phe 2.00(2), Lys 8.44(8), His 2.31(3), Arg 2.84(3), average recovery 86% (Found: C, 53.00; H, 6.24; N, 12.95. $C_{701}H_{955}N_{143}O_{200}S_{14} \cdot 43 H_2O$, M.W. 15033.690 + hydration, requires C, 53.26; H, 6.64; N, 12.67%).

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Z(OMe)-Ala-Ala-NHNH-Troc. ————— Z(OMe)-Ala-NHNH-Troc
(37.0 g, 84 mmol) was treated with TFA-anisole (50 ml-18ml) as usual, then excess TFA was removed by evaporation. The oily residue was washed with n-hexane, dried over KOH pellets in vacuo for 3 hr and dissolved in DMF (200 ml) together with Et_3N (23.5 ml, 0.17 mol) and Z(OMe)-Ala-ONP (36.02 g, 96 mmol). After stirring for 48 hr, the solution was concentrated and the residue was purified by procedure A followed by recrystallization from AcOEt and ether to give the dipeptide derivative (36.04 g, 91%), m.p. 118-120°, $[\alpha]_D^{27}$ - 11.5° (c 1.4 in DMF), R_{f1} 0.63 (Found: C, 42.31; H, 4.52; N, 10.69. $C_{18}H_{23}N_4O_7Cl_3$ requires C, 42.08; H, 4.51; N, 10.91%).

Z(OMe)-Ser-Ala-Ala-NHNH-Troc. ————— Z(OMe)-Ala-Ala-NHNH-Troc
(41.03 g, 80 mmol) was treated with TFA-anisole (40 ml-15 ml), then excess TFA was removed by evaporation. The oily residue, isolated as mentioned above, was dissolved in DMF (250 ml) together with Et_3N (22.05 ml, 0.16 mol) and Z(OMe)-Ser-OPCP (41.36 g, 80 mmol). After stirring for 48 hr, the solution was concentrated and the residue was purified by procedure A followed by recrystallization from MeOH to give the protected tripeptide derivative (34.60 g, 72%), m.p. 179-183°, $[\alpha]_D^{25}$ -3.8° (c 0.8 in DMF), R_{f1} 0.53 (Found: C, 42.02; H, 4.65; N, 11.76. $C_{21}H_{28}N_5O_9Cl_3$ requires C,

41.98; H, 4.70; N, 11.66%).

Z(OMe)-Ser-Thr-OMe. ——— The azide (prepared from 32.76 g, 0.12 mol of Z(OMe)-Ser-NHNH₂) in DMF (200 ml) and Et₃N (15.95 ml, 0.12 mol) were added to an ice-chilled solution of H-Thr-OMe (prepared from 18.01 g, 0.12 mol of the hydrochloride with 15.97 ml, 0.12 mol of Et₃N) in DMF (150 ml). After stirring for 24 hr, the solution was concentrated and the residue was purified by procedure A followed by recrystallization from MeOH and ether to give the protected dipeptide (21.20 g, 48%), m.p. 125-127°, $[\alpha]_D^{30} + 7.2^\circ$ (c 1.0 in DMF), Rf₁ 0.66 (Found: C, 53.09; H, 6.50; N, 7.27. C₁₇H₂₄N₂O₈ requires C, 53.12; H, 6.29; N, 7.29%).

Z(OMe)-Ser-Thr-NHNH₂. ——— Z(OMe)-Ser-Thr-OMe (13.20 g, 34 mmol) in MeOH (80 ml) was treated with 80% hydrazine hydrate (17 ml, 8 equiv.) overnight. The resulting mass was precipitated from DMF with MeOH to yield the hydrazide (11.05 g, 85%), m.p. 204-207°, $[\alpha]_D^{25} + 10.5^\circ$ (c 0.7 in DMSO), Rf₁ 0.50 (Found: C, 49.86; H, 6.40; N, 14.67. C₁₆H₂₃N₃O₇ requires C, 49.99; H, 6.29; N, 14.58 %).

Z(OMe)-Ser-Thr-Ser-Ala-Ala-NHNH-Troc. ——— Z(OMe)-Ser-Ala-Ala-NHNH-Troc (14.77 g, 25 mmol) was treated with TFA-anisole (42 ml-14 ml) as usual, then dry ether was added. The resulting powder was dried over KOH pellets in vacuo for 3 hr and dissolved in DMF (120 ml) containing Et₃N (3.39 ml, 25 mmol). To this ice-chilled solution, the azide (prepared from 10.42 g, 27 mmol of Z(OMe)-Ser-Thr-NHNH₂) in DMF (70 ml) and Et₃N (3.74 ml, 27 mmol) were added and the solution, after stirring for 48 hr, was concentrated. The residue was purified by procedure B followed by precipitations from DMF with MeOH (2x) to yield the protected pentapeptide derivative (17.15 g, 89%), m.p. 224-228°, $[\alpha]_D^{25} - 7.5^\circ$ (c 1.6 in DMSO), Rf₁

0.32. Amino acid analysis: Ser 1.39, Thr 0.85, Ala 2.00, average recovery 91% (Found: C, 42.67; H, 5.19; N, 12.63; $C_{28}H_{40}N_7O_{13}Cl_3$ requires C, 42.62; H, 5.11; N, 12.43%).

Z(OMe)-Ser-Ser-Thr-Ser-Ala-Ala-NHNH-Troc. ——— Z(OMe)-Ser-Thr-Ser-Ala-Ala-NHNH-Troc (17.15 g, 22 mmol) was treated with TFA-anisole (50 ml-17 ml) and the N^α -deprotected peptide, isolated as mentioned above, was dissolved in DMF (150 ml) containing Et_3N (3.0 ml, 22 mmol). To this ice-chilled solution, the azide (prepared from 7.39 g, 26 mmol of Z(OMe)-Ser-NHNH₂) in DMF (70 ml) and Et_3N (3.6 ml, 26 mmol) were added. After stirring for 48 hr, the solution was concentrated and the residue was purified by procedure B followed by precipitation from DMF with MeOH (2x) to yield the protected hexapeptide derivative (16.99 g, 89%), m.p. 241-244°, $[\alpha]_D^{25} - 7.4^\circ$ (c 1.1 in DMF), Rf_1 0.41. Amino acid analysis: Ser 2.46, Thr 0.94, Ala 2.00, average recovery 80% (Found: C, 42.47; H, 5.42; N, 12.71. $C_{31}H_{45}N_8O_{15}Cl_3$ requires C, 42.50; H, 5.18; N, 12.79%).

Z(OMe)-Asp(OBu^t)-Ser-Ser-Thr-Ser-Ala-Ala-NHNH-Troc, Z(OMe)-(RNase 14-20)-NHNH-Troc [A]. ——— Z(OMe)-Ser-Ser-Thr-Ser-Ala-Ala-NHNH-Troc (9.33 g, 10.7 mmol) was treated with TFA-anisole (40 ml-10 ml) and the N^α -deprotected peptide, isolated as mentioned above, was dissolved in DMSO-DMF (1:1, 100 ml) together with Et_3N (2.94 ml, 21.3 mmol) and Z(OMe)-Asp(OBu^t)-ONP (5.05 g, 10.7 mmol). After stirring for 24 hr, the solution was concentrated and the residue was purified by procedure B followed by precipitation from DMF with MeOH (2x) to give the protected heptapeptide derivative (8.77 g, 79%), m.p. 221° dec., $[\alpha]_D^{20} - 9.2^\circ$ (c 0.9 in DMSO), Rf_1 0.19, Rf_3 0.82. Amino acid analysis: Asp 1.00, Ser 2.24, Thr 0.86, Ala 2.00, average recovery 93% (Found: C, 44.38; H, 5.54; N, 11.65. $C_{39}H_{58}N_9O_{18}Cl_3 \cdot 1/2 H_2O$ requires C, 44.34; H, 5.63; N, 11.93%).

Z(OMe)-Met(O)-NHNH-Troc. ————— DCC (10.89 g, 53 mmol) was added to a stirred solution of Z(OMe)-Met(O)-OH (15.84 g, 48 mmol) and Troc-NHNH₂ (10.97 g, 53 mmol) in THF (200 ml). After 24 hr, the filtered solution was concentrated and the residue was purified by procedure A followed by recrystallization from AcOEt to give the hydrazide derivative (16.22 g, 65%), m.p. 133-138°, $[\alpha]_D^{25} + 12.9^\circ$ (c 1.0 in DMF), Rf_2 0.56 (Found: C, 39.25; H, 4.23; N, 8.01. C₁₇H₂₂N₃O₇SCl₃ requires C, 39.35; H, 4.27; N, 8.10%).

Z(OMe)-Gln-His-Met(O)-NHNH-Troc. ————— Z(OMe)-Met(O)-NHNH-Troc (10.63 g, 20.5 mmol) was treated with TFA-anisole (40 ml-10 ml) as usual, then n-hexane was added. The resulting oily precipitate was dried over KOH pellets in vacuo for 3 hr and dissolved in DMF (100 ml) containing Et₃N (2.83 ml, 20.5 mmol). To this ice-chilled solution, the azide (prepared from 9.46 g, 20.5 mmol of Z(OMe)-Gln-His-NHNH₂⁹⁷) in DMF (80 ml) and Et₃N (2.83 ml, 20.5 mmol) were added and the mixture, after stirring for 48 hr, was concentrated. The residue was dissolved in n-BuOH saturated previously with H₂O. The organic phase was washed three times with 5% Na₂CO₃ and H₂O, dried over MgSO₄ and concentrated. The residue was washed with H₂O and recrystallization from EtOH to give the protected tripeptide derivative (12.25 g, 76%), m.p. 142-146°, $[\alpha]_D^{25} + 6.5^\circ$ (c 1.1 in DMF), Rf_1 0.49 (Found: C, 42.99; H, 5.04; N, 14.45. C₂₈H₃₇N₈O₁₀SCl₃ requires C, 42.89; H, 4.76; N, 14.29%).

Z(OMe)-Arg(MBS)-Gln-His-Met(O)-NHNH-Troc. ————— Z(OMe)-Gln-His-Met(O)-NHNH-Troc (10.58 g, 13.5 mmol) was treated with TFA-anisole (30 ml-10 ml), then dry ether was added. The resulting powder, isolated as mentioned previously, was dissolved in DMF (100 ml) containing Et₃N (3.72 ml, 27 mmol). To this ice-chilled solution, the mixed anhydride (prepared from 8.23 g, 16.2 mmol of Z(OMe)-Arg(MBS)-OH) in dry THF (60 ml) was added. After stirring

in an ice-bath for 3 hr, the solution was concentrated and the residue was purified by washing 5% NaHCO_3 and H_2O followed by precipitation from DMF with MeOH to afford the protected tetrapeptide derivative (10.72 g, 72%), m.p. 176-181°, $[\alpha]_D^{25} - 18.0^\circ$ (c 0.7 in DMF), Rf_1 0.53 (Found: C, 44.98; H, 5.21; N, 15.38. $\text{C}_{41}\text{H}_{55}\text{N}_{12}\text{O}_{14}\text{S}_2\text{Cl}_3$ requires C, 44.34; H, 4.99; N, 15.14%).

Z(OMe)-Glu(OBzl)-Arg(MBS)-Gln-His-Met(O)-NHNH-Troc [B].

Z(OMe)-Arg(MBS)-Gln-His-Met(O)-NHNH-Troc (11.69 g, 10.5 mmol) was treated with TFA-anisole (40 ml-12 ml) and the N^α -deprotected peptide, isolated as mentioned above, was dissolved in DMF (100 ml) together with Et_3N (4.36 ml, 31.6 mmol) and Z(OMe)-Glu(OBzl)-ONP (5.50 g, 10.5 mmol). After stirring for 24 hr, the solution was concentrated and the residue was purified by procedure B followed by precipitation from DMF with EtOH to yield the protected pentapeptide derivative (10.99 g, 79%), m.p. 192-197°, $[\alpha]_D^{25} - 14.4^\circ$ (c 0.7 in DMF), Rf_1 0.51. Amino acid analysis: Glu 2.08, Arg 0.96, His 1.00, Met + Met(O) 0.75, average recovery 89% (Found: C, 46.03; H, 5.08; N, 13.04. $\text{C}_{53}\text{H}_{68}\text{N}_{13}\text{O}_{17}\text{S}_2\text{Cl}_3 \cdot 2.5 \text{H}_2\text{O}$ requires C, 46.30; H, 5.35; N, 13.25%).

Z(OMe)-Glu(OBzl)-Arg(MBS)-Gln-His-Met(O)-NHNH₂, Z(OMe)-(RNase 9-13)-NHNH₂. The above protected pentapeptide (10.54 g, 7.9 mmol) in DMF-AcOH (100 ml-20 ml) was treated with Zn powder (5.18 g, 10 equiv.) in an ice-bath for 10 min and at room temperature for 3 hr. The solution was filtered, the filtrate was concentrated in vacuo at the bath temperature (below 35°), and the residue was treated with a saturated solution of EDTA and 5% NaHCO_3 . The resulting mass was washed with H_2O and precipitated twice from DMSO with MeOH to afford the hydrazide (6.72 g, 73%), m.p. 229 - 236°, $[\alpha]_D^{20} - 22.5^\circ$ (c 0.9 in DMSO), Rf_3 0.47. Amino acid analysis: Glu 2.00, Arg 1.06, His 1.01, Met + Met(O) 0.68, average re-

covery 93% (Found: C, 50.32; H, 6.22; N, 14.91. $C_{50}H_{67}N_{13}O_{15}S_2 \cdot 2.5 H_2O$ requires C, 50.07; H, 6.05; N, 15.18%).

Z(OMe)-Lys(Z)-Phe-OMe. ——— DCC (22.66 g, 0.11 mol) was added to a stirred mixture of Z(OMe)-Lys(Z)-OH (44.45 g, 0.10 mol) and H-Phe-OMe (prepared from 21.52 g, 0.10 mol of the hydrochloride with 13.8 ml, 0.10 mol of Et_3N) in DMF (200 ml). After 48 hr, the filtered solution was concentrated and the residue was purified by procedure B followed by recrystallization from MeOH and AcOEt to give the protected dipeptide ester (43.33 g, 72%), m.p. 129-131°, $[\alpha]_D^{22} - 3.6^\circ$ (c 1.1 in DMF), Rf_1 0.92 (Found: C, 65.35; H, 6.58; N, 7.18. $C_{33}H_{39}N_3O_8$ requires C, 65.44; H, 6.49; N, 6.94%).

Z(OMe)-Ala-Lys(Z)-Phe-OMe. ——— Z(OMe)-Lys(Z)-Phe-OMe (40.01 g, 66 mmol) was treated with TFA-anisole (40 ml-20 ml) as usual, then n-hexane was added. The resulting oily precipitate, isolated as mentioned previously, was dissolved in DMF (250 ml) together with Et_3N (18.2 ml, 0.13 mol) and Z(OMe)-Ala-ONP (26.20 g, 70 mmol) and the mixture, after stirring for 48 hr, was concentrated. The residue was purified by procedure B followed by recrystallization from dioxane and MeOH to afford the protected tripeptide ester (35.65 g, 80%), m.p. 159-162°, $[\alpha]_D^{25} - 6.9^\circ$ (c 1.5 in DMF), Rf_1 0.85 (Found: C, 63.97; H, 6.49; N, 7.99. $C_{36}H_{44}N_4O_9$ requires C, 63.89; H, 6.55; N, 8.28%).

Z(OMe)-Ala-Ala-Lys(Z)-Phe-OMe. ——— Z(OMe)-Ala-Lys(Z)-Phe-OMe (33.02 g, 49 mmol) was treated with TFA-anisole (33 ml-16 ml) as usual, then dry ether was added. The resulting powder, isolated as mentioned above, was dissolved in DMF (250 ml) together with Et_3N (13.5 ml, 98 mmol) and Z(OMe)-Ala-ONP (18.70 g, 50 mmol) and the mixture, after stirring for 48 hr, was concentrated. The residue was purified by procedure B followed by precipitation from DMF

with MeOH to afford the protected tetrapeptide ester (27.32 g, 75%), m.p. 203-204°, $[\alpha]_D^{25} - 9.8^\circ$ (c 0.6 in DMF), Rf_1 0.90 (Found: C, 62.51; H, 6.48; N, 9.44. $C_{39}H_{49}N_5O_{10}$ requires C, 62.63; H, 6.60; N, 9.37%).

Z(OMe)-Thr-Ala-OMe. ————— Z(OMe)-Thr-OPCP (37.11 g, 70 mmol) was added to a stirred solution of H-Ala-OMe (prepared from 9.77 g, 70 mmol of the hydrochloride with 9.64 ml, 70 mmol of Et_3N) in DMF (100 ml). After 48 hr, the solvent was evaporated and the residue was purified by procedure B followed by recrystallization from AcOEt and ether to afford the protected dipeptide ester (18.49 g, 72%), m.p. 123-126°, $[\alpha]_D^{25} - 2.6^\circ$ (c 0.8 in DMF), Rf_1 0.80 (Found: C, 55.72; H, 6.81; N, 7.72. $C_{17}H_{24}N_2O_7$ requires C, 55.42; H, 6.57; N, 7.61%).

Z(OMe)-Thr-Ala-NHNH₂. ————— Z(OMe)-Thr-Ala-OMe (21.03 g, 57 mmol) in MeOH (150 ml) was treated with 80% hydrazine hydrate (18 ml, 5 equiv.) overnight. The resulting mass was recrystallized from MeOH to afford the hydrazide (18.48 g, 88%), m.p. 199-200°, $[\alpha]_D^{25} + 7.1^\circ$ (c 0.7 in DMSO), Rf_1 0.64 (Found: C, 51.60; H, 6.54; N, 14.99. $C_{16}H_{24}N_4O_6$ requires C, 52.16; H, 6.57; N, 15.21%).

Z(OMe)-Thr-Ala-Ala-Ala-Lys(Z)-Phe-OMe. ————— Z(OMe)-Ala-Ala-Lys(Z)-Phe-OMe (7.70 g, 10.3 mmol) was treated with TFA-anisole (30 ml-7 ml) and the N^α -deprotected peptide, isolated as mentioned above, was dissolved in DMF (70 ml) containing Et_3N (14.2 ml, 10.3 mmol). To this ice-chilled solution, the azide (prepared from 5.30 g, 14.4 mmol of Z(OMe)-Thr-Ala-NHNH₂) in DMF (30 ml) and Et_3N (1.99 ml, 14.4 mmol) were added and the mixture was stirred for 48 hr. The solvent was evaporated and the residue was purified by procedure B followed by precipitation from DMF with MeOH (2x) to afford the protected hexapeptide (8.52 g, 90%), m.p. 218-222°, $[\alpha]_D^{25}$

- 10.1° (c 0.7 in DMF), R_{f1} 0.73. Amino acid analysis: Thr 0.93, Ala 2.89, Lys 1.03, Phe 1.00, average recovery 91% (Found: C, 59.79; H, 6.68; N, 10.54. $C_{46}H_{61}N_7O_{13}$ requires C, 60.05; H, 6.68; N, 10.66%).

Z-Lys(Z)-Glu(OBu^t)-OH. ——— Z-Lys(Z)-OSu (12.48 g, 24 mmol) in THF (120 ml) was added to a stirred solution of H-Glu(OBu^t)-OH (4.92 g, 24 mmol) and Et₃N (6.68 ml, 48 mmol) in H₂O (50 ml). After 24 hr, the solvent was evaporated and the residue was extracted with AcOEt. The organic phase was washed with 5% citric acid and H₂O-NaCl, dried over Na₂SO₄ and concentrated. Trituration with ether and n-hexane followed by recrystallization from AcOEt and n-hexane afforded the protected dipeptide (12.23 g, 84%), m.p. 86-89°, $[\alpha]_D^{25}$ - 4.3° (c 0.7 in DMF), R_{f1} 0.68 (Found: C, 62.16; H, 6.96; N, 6.97. $C_{31}H_{41}N_3O_9$ requires C, 62.09; H, 6.89; N, 7.01%).

Z-Lys(Z)-Glu(OBu^t)-OMe. ——— An ethereal solution of diazomethane was added to an ice-chilled solution of Z-Lys(Z)-Glu(OBu^t)-OH (8.10 g, 13.5 mmol) in MeOH (80 ml), until the yellow colour persisted for 30 min. After addition of a few drops of AcOH, the solvent was removed by evaporation. Trituration with ether followed by recrystallization from AcOEt and n-hexane afforded the dipeptide ester (7.12 g, 86%), m.p. 64-67°, $[\alpha]_D^{25}$ - 5.9° (c 0.9 in DMF), R_{f1} 0.97 (Found: C, 62.35; H, 7.00; N, 6.81. $C_{32}H_{43}N_3O_9$ requires C, 62.62; H, 7.06; N, 6.85%).

Z-Lys(Z)-Glu(OBu^t)-NHNH₂. ——— Z-Lys(Z)-Glu(OBu^t)-OMe (7.11 g, 11.6 mmol) in EtOH (70 ml) was treated with 80% hydrazine hydrate (3.63 ml, 5 equiv.) at 4° for 48 hr, then ether was added. The resulting gelatinous mass was recrystallized from MeOH and ether to afford the hydrazide (5.72 g, 80%), m.p. 101-105°, $[\alpha]_D^{25}$ - 7.7° (c 1.0 in DMF), R_{f1} 0.73 (Found: C, 60.07; H, 7.00; N,

11.85. $C_{31}H_{43}N_5O_8$ requires C, 60.67; H, 7.06; N, 11.41%.

Z-Lys(Z)-Glu(OBu^t)-Thr-Ala-Ala-Ala-Lys(Z)-Phe-OMe. ————
Z(OMe)-Thr-Ala-Ala-Ala-Lys(Z)-Phe-OMe (7.74 g, 8.4 mmol) was treated with TFA-anisole (32 ml-8 ml) as usual, then dry ether was added. The resulting powder, isolated as mentioned above, was dissolved in DMSO-DMF (1:1, 80 ml) containing Et₃N (1.17 ml, 8.4 mmol). To this ice-chilled solution, the azide (prepared from 6.22 g, 10.1 mmol of Z-Lys(Z)-Glu(OBu^t)-NHNH₂) in DMF (30 ml) and Et₃N (1.40 ml, 10.1 mmol) were added and the mixture, after stirring for 48 hr, was concentrated. The residue was purified by procedure B followed by precipitation from DMSO with MeOH to afford the protected octapeptide ester (9.67 g, 86%), m.p. 233-236°, $[\alpha]_D^{25}$ -12.0° (c 0.8 in DMF), Rf₁ 0.76, Rf₆ 0.69. Amino acid analysis: Lys 2.02, Glu 1.00, Thr 0.89, Ala 2.86, Phe 1.00, average recovery 87% (Found: C, 60.85; H, 6.88; N, 10.34. $C_{68}H_{92}N_{10}O_{18}$ requires C, 61.06; H, 6.93; N, 10.47%).

Z-Lys(Z)-Glu(OBu^t)-Thr-Ala-Ala-Ala-Lys(Z)-Phe-NHNH₂, Z-
(RNase 1-8)-NHNH₂ [C]. ———— The above protected octapeptide ester (7.21 g, 5.4 mmol) dissolved in HMPA-MeOH (4:1, 70 ml) was treated with 80% hydrazine hydrate (1.69 ml, 5 equiv.) for 24 hr, then H₂O (300 ml) was added. The resulting powder was washed with H₂O and precipitated from DMSO with MeOH to yield the protected octapeptide hydrazide (6.31 g, 88%), m.p. 234-238°, $[\alpha]_D^{20}$ -15.1° (c 1.1 in DMSO), Rf₃ 0.86, Rf₅ 0.28. Amino acid analysis: Lys 2.00, Glu 1.01, Thr 0.96, Ala 2.90, Phe 1.00, average recovery 92% (Found: C, 59.96; H, 6.99; N, 12.30. $C_{67}H_{92}N_{12}O_{17}$ requires C, 60.16; H, 6.93; N, 12.57%).

Z(OMe)-Glu(OBzl)-Arg(MBS)-Gln-His-Met(O)-Asp-Ser-Ser-Thr-Ser-
Ala-Ala-NHNH-Troc. ———— Z(OMe)-(RNase 14-20)-NHNH-Troc [A]

(4.28 g, 4.1 mmol) was treated with TFA-anisole (16 ml-4 ml) in an ice-bath for 3 hr and at room temperature for 30 min, then dry ether was added. The resulting powder, isolated as mentioned above, was dissolved in DMSO-DMF (1:1, 40 ml) containing Et₃N (1.13 ml, 8.2 mmol). To this ice-chilled solution, the azide (prepared from 4.71 g, 4.1 mmol of Z(OMe)-(RNase 9-13)-NHNH₂) in DMSO-DMF (1:1, 30 ml) and Et₃N (0.56 ml, 4.1 mmol) were added and the solution, after stirring for 24 hr, was concentrated. The residue was purified by procedure B followed by precipitation from DMF with AcOEt (2x) to afford the protected dodecapeptide derivative (6.14 g, 77%), m.p. 200-203°, $[\alpha]_D^{25} - 13.6^\circ$ (c 0.9 in DMSO), Rf₃ 0.66. Amino acid analysis: Asp 1.04, Thr 0.89, Ser 2.35, Glu 2.11, Ala 2.00, Met + Met(O) 0.93, His 1.05, Arg 1.04, average recovery 88% (Found: C, 46.13; H, 5.82; N, 13.63. C₇₆H₁₀₅N₂₀O₃₀S₂Cl₃ · 2.5 H₂O requires C, 45.77; H, 5.56; N, 14.04%).

Z-Lys(Z)-Glu(OBu^t)-Thr-Ala-Ala-Ala-Lys(Z)-Phe-Glu(OBzl)-Arg(MBS)-Gln-His-Met(O)-Asp-Ser-Ser-Thr-Ser-Ala-Ala-NHNH-Troc.

———— The above protected dodecapeptide (1.24 g, 0.64 mmol) was treated with TFA-anisole (4 ml-1 ml) and the N^α-deprotected peptide, isolated as mentioned above, was dissolved in DMSO-DMF (1:1, 7 ml) containing Et₃N (0.18 ml, 1.27 mmol) and N-methylmorpholine (0.07 ml, 0.64 mmol). To this ice-chilled solution, the azide (prepared from 0.85 g, 0.64 mmol of Z-(RNase 1-8)-NHNH₂ [C]) in DMSO-DMF (1:1, 6 ml) and N-methylmorpholine (0.07 ml, 0.64 mmol) were added. After stirring for 48 hr, the solution was concentrated and the residue was purified by procedure B followed by precipitation from DMSO with MeOH (2x) to afford the protected docosapeptide derivative (1.02 g, 52%), m.p. 228° dec., $[\alpha]_D^{25} - 13.7^\circ$ (c 0.7 in DMSO), Rf₃ 0.76. Amino acid analysis: Asp 0.94, Thr 1.77, Ser 2.22, Glu 2.98, Ala 5.10, Met + Met(O) 0.62, Phe 1.00, Lys 2.08, His 0.90, Arg 0.95, average recovery 92% (Found: 51.47; H, 6.10; N,

13.23. $C_{134}H_{185}N_{30}O_{44}S_2Cl_3 \cdot 2 H_2O$ requires C, 51.47; H, 6.09; N, 13.44%).

Z-Lys(Z)-Glu(OBu^t)-Thr-Ala-Ala-Ala-Lys(Z)-Phe-Glu(OBzl)-Arg(MBS)-Gln-His-Met(O)-Asp-Ser-Ser-Thr-Ser-Ala-Ala-NHNH₂, Z-(RNase 1-20)-NHNH₂, Protected S-peptide.

The above protected eicosapeptide derivative (0.53 g, 0.17 mmol) in HMPA-NMP (2:1, 5 ml) and AcOH (1 ml) was treated with Zn powder (0.56 g, 50 equiv.) at 40° for 3 hr. The solvent was removed by evaporation and the residue was treated with 5% EDTA. The resulting gelatinous mass was purified by washing with H₂O followed by precipitation from DMSO with MeOH to yield the hydrazide (0.41 g, 83%), m.p. 251° dec., $[\alpha]_D^{20}$ - 28.9° (c 0.8 in DMSO), R_f 0.61. Amino acid analysis: Asp 0.93, Thr 1.74, Ser 2.09, Glu 3.02, Ala 4.85, Met + Met(O) 0.77, Phe 1.00, Lys 2.13, His 0.91, Arg 0.91, average recovery 93% (Found: C, 53.42; H, 6.52; N, 13.30. $C_{131}H_{184}N_{30}O_{42}S_2 \cdot 3 H_2O$ requires C, 52.99; H, 6.45; N, 14.15%).

Section 6. Synthesis of RNase A with the Full Enzymic Activity

Condensation of Z-(RNase 1-20)-NHNH₂ and H-(RNase 21-124)-OBzl.

———— Z(OMe)-(RNase 21-124)-OBzl (213 mg, 14.2 μ mol) was treated with TFA-anisole (1.5 ml-0.3 ml) in an ice-bath for 90 min, then dry ether was added. The resulting powder was washed with ether, dried over KOH pellets in vacuo for 3 hr and dissolved in DMSO-HMPA (1:1, v/v, 1 ml) containing 10% Et₃N in DMF (79 μ l, 56.8 μ mol). To this ice-chilled solution, the azide (prepared from 253 mg, 86.9 μ mol of Z-(RNase 1-20)-NHNH₂) in DMF-DMSO-HMPA (1:1:1, 1.5 ml) and 10% Et₃N in DMF (0.12 ml, 86.9 μ mol) were added and the mixture was stirred at 4° for 72 hr. An additional azide (prepared from the same amount of the hydrazide) in DMF-DMSO-HMPA (1.5 ml) and 10% Et₃N in DMF (0.12 ml) were added and stirring was further continued for 72 hr. Although the solution still remained positive to ninhydrin, H₂O (60 ml) was added. The resulting powder was collected by filtration, washed with 3% AcOH, 5% NaHCO₃ and H₂O and precipitated from DMSO with MeOH. The product was dissolved in 5% H₂O-DMSO and the solution was applied to a column of Sephacryl S-200 (3 x 142 cm), which was eluted with the same solvent. Individual fractions (5.1 ml each) were collected and absorption at 275 nm was determined. Two peaks were detected; Peak 1 (154 mg, tube Nos. 55-75, a mixture of the protected RNase and the unreacted amino component) Peak 2 (299 mg, tube Nos. 116-131, the rearrangement product of the azide). Amino acid ratios in acid hydrolysate of the peak 1 substance: Asp 14.46, Thr 8.15, Ser 10.74, Glu 10.19, Pro 3.74, Gly 3.00, Ala 8.17, Val 8.64, Met + Met(O) 2.66, Ile 1.96, Leu 2.00, Tyr 6.08, Phe 2.22, Lys 8.91, His 2.96, Arg 3.27 (recovery of Leu 85%). Incorporation of new amino acids was ca. 22%.

Z(OMe)-Glu(OBzl)-Arg(MBS)-Gln-His-Met(O)-Asp-Ser-Ser-Thr-Ser-Ala-Ala-NHNH₂, Z(OMe)-(RNase 9-20)-NHNH₂ [29]. —————
 Z(OMe)-(RNase 9-20)-NHNH-Troc (4.77 g, 2.45 mmol) dissolved in a mixture of DMF-HMPA (1:4, 50 ml) and AcOH (10 ml) was treated with Zn (1.6 g, 10 equiv.) at room temperature for 5 hr, while the starting material disappeared on tlc. Some material precipitated during the reaction was dissolved by a slight warming at 40° and the solution was filtered. The filtrate was concentrated and H₂O (200 ml) was added. The resulting oily precipitate was treated with a saturated solution of EDTA. Storage in a refrigerator afforded a powder, which was precipitated from DMSO with MeOH to afford the protected dodecapeptide hydrazide (3.37 g, 78%), m.p. 246° dec., $[\alpha]_D^{25} - 17.2^\circ$ (c 0.9 in DMSO), R_f_3 0.62. Amino acid ratios in acid hydrolysate: Glu 2.08, Arg 1.10, His 1.12, Met + Met(O) 0.74, Asp 1.03, Ser 2.74, Thr 0.97, Ala 2.00, average recovery 91%. (Found: C, 48.30; H, 5.98; N, 14.72. C₇₃H₁₀₄N₂₀O₂₈S₂ · 3 H₂O requires C, 47.96; H, 6.07; N, 15.33%).

Z(OMe)-(RNase 9-124)-OBzl, [Z(OMe)-Glu(OBzl)-Arg(MBS)-Gln-His-Met(O)-Asp-Ser-Ser-Thr-Ser-Ala-Ala-(21-124)-OBzl]. —————
 ————— Z(OMe)-(RNase 21-124)-OBzl (829 mg, 55.1 μmol) was treated with TFA-anisole (6 ml-1 ml) and the N^α-deprotected peptide, isolated as mentioned above, was dissolved in DMSO-HMPA (1:1, 6 ml) containing Et₃N (30 μl, 220 μmol). To this ice-chilled solution, the azide (prepared from 977 mg, 10 equiv. of Z(OMe)-(RNase 9-20)-NHNH₂) in DMSO-DMF (1:1, 4 ml) and Et₃N (76 μl, 551 μmol) were added and the mixture was stirred at 4° for 72 hr. Three additions of the azide (10, 5 and 5 equiv.) were carried out in 48 and 24 hr intervals. After the final addition of the azide, the mixture was further stirred for 24 hr, while the ninhydrin intensity on the origin of the tlc plate disappeared, when examined by a shimadzu dual wavelength tlc scanner. The mixture was then stir-

red at room temperature for 6 hr. Addition of H₂O (60 ml) afforded a powder, which was collected by filtration, washed with 3% AcOH and precipitated from DMSO with MeOH to give the crude product (2.41 g). The crude product (500 mg each), dissolved in 5% H₂O-DMSO (5 ml), was applied to a column of Sephacryl S-200 (5 x 126 cm), which was eluted with the same solvent at a flow rate of 72 ml/hr. Individual fractions (15 ml each) were collected and absorption at 275nm was determined. Fractions of the front peak (Fig. 5-a, tube Nos. 56-80) were combined, the solvent was removed by evaporation and the residue was precipitated from DMSO with MeOH to give a powder (620 mg, 68% in total yield of 5 purifications), m.p. 242° dec., $[\alpha]_D^{25} = -26.8^\circ$ (c 0.7 in DMSO), $R_f_3 = 0$ (Found: C, 51.88; H, 5.84; N, 12.39. C₇₆₅H₁₀₄₇N₁₆₁O₂₂₅S₁₆ · 64 H₂O requires C, 51.72; H, 6.67; N, 12.70%).

Z-(RNase 1-124)-OBzl, [Z-Lys(Z)-Glu(OBu^t)-Thr-Ala-Ala-Ala-Lys(Z)-Phe-(9-124)-OBzl], Protected RNase.

Z(OMe)-(RNase 9-124)-OBzl (616 mg, 37.1 μmol) was treated with TFA-anisole (6 ml-1 ml) and the N^α-deprotected peptide, isolated as mentioned above, was dissolved in DMSO-HMPA (1:1, 5 ml) containing Et₃N (31 μl, 223 μmol). To this ice-chilled solution, the azide (prepared from 658 mg, 10 equiv. of Z-(RNase 1-8)-NHNH₂) in DMSO-DMF (1:1, 2 ml) and N-methylmorpholine (41 μl, 371 μmol) were added and the mixture was stirred at 4° for 72 hr. Three additions of the azide (10, 5 and 5 equiv.) were carried out in 48 and 24 hr intervals. After the final addition of the azide, stirring was continued for an additional 24 hr. The solution was then stirred at room temperature for 6 hr and H₂O (100 ml) was added. The resulting powder was collected by filtration, washed with 3% AcOH and precipitated twice from DMSO with MeOH to give the crude product. The product was purified by column chromatography on Sephacryl S-200 (5 x 132 cm) with 5% H₂O DMSO as eluate. Individual fractions (15 ml

each) were collected and absorption at 275 nm was determined. Fractions corresponding to the front peak (Fig. 5-b, tube Nos. 60-85) were collected and the solvent was removed by evaporation. Treatment of the residue with MeOH afforded a powder (505 mg, 77%), m.p. 233° dec., $[\alpha]_D^{25} - 25.1^\circ$ (c 0.5 in DMSO), $Rf_3 = 0$ (Found: C, 53.14; H, 6.44; N, 12.82. $C_{823}H_{1127}N_{171}O_{239}S_{16} \cdot 42 H_2O$ requires C, 53.40; H, 6.59; N, 12.94%).

Reduction of the sulphoxide of Cys(MBzl) and Met residue of the protected RNase.

Thiophenol (3.72 ml, 1280 equiv.) was added to a solution of the protected RNase (502 mg, 28.3 μ mol) in HMPA-NMP (2:1, 10 ml) and, under N_2 atmosphere, the solution was incubated at 90° for 25 hr, while the standard samples of Z(OMe)-Cys(MBzl)(O)-OH and Z(OMe)-Met(O)-OH were completely reduced under identical conditions. It was confirmed that Z(OMe)-Asp(OBzl)-OH and Z(OMe)-Glu(OBzl)-OH remained intact under these conditions. The solvent was removed by evaporation and the residue was treated with MeOH. The resulting powder was precipitated from HMPA with MeOH to give the reduced protected RNase (452 mg, 90%), m.p. 239° dec., $[\alpha]_D^{25} - 24.4^\circ$ (c 0.7 in DMSO). Recoveries of cystine in acid hydrolysates of the protected RNase, before and after reduction, were 2.82 and 3.50 (theory 4).

[A] Deprotection of the protected RNase by MSA.

Under an N_2 atmosphere, the reduced RNase, Z-(RNase 1-124)-OBzl (100 mg, 5.63 μ mol) was treated with freshly distilled MSA (3.9 ml) in the presence of m-cresol (0.39 ml, 660 equiv.) in an ice-bath for 10 min and at 25° for 60 min. Dry ether was added and the resulting powder was retreated with MSA under identical conditions as mentioned above. The deprotected peptide, precipitated by ether, was then dissolved in a 0.2 M Tris-HCl buffer (3 ml) containing

4M guanidine HCl and the pH of the solution was adjusted to 8.6 with 5% methylamine in H_2O . After addition of 2-mercaptoethanol (0.35 ml, 800 equiv.) and dithiothreitol (174 mg, 200 equiv.), the solution was incubated, under N_2 , at 50° for 24 hr, while a marker, S-methyl-methionine, was completely reduced to methionine. 2-mercaptoethanol (0.18 ml, 400 equiv.) was further added and the solution was incubated for an additional 40 hr. The pH of the solution was then adjusted to 3 with 3N HCl and the solution was applied to a column of Sephadex G-25 (1.6 x 90 cm), which was eluted with 0.1 N AcOH. Individual fractions (4 ml each) were collected and absorptions at 275 nm were determined. Fractions (tube Nos. 23-40) containing the reduced substance were then subjected to air oxidation.

Air oxidation of the deprotected peptide. _____

Fractions (tube Nos. 23-40) obtained from gel-filtration on Sephadex G-25 were combined and the solution was diluted with H_2O (3 l) and the entire volume was brought up to 4 l with 0.2M Tris-HCl buffer at pH 8.2. The protein concentration of this solution was 0.02 mg/ml. The solution was kept standing at room temperature for 2 days, during which time the Ellman test dropped from 0.097 to the constant value, 0.001. After adjusting the pH to 3.0 with 2N HCl, the entire solution was lyophilized. The residue was dialyzed with a cellulose tubing against distilled H_2O (1 l) three times for desalting and the solution was lyophilized to give a hygroscopic powder (169 mg). The crude oxidized substance was dissolved in 0.05M NH_4HCO_3 , pH 8.4 (2 ml) and some insoluble material (ca. 6 mg) was removed by centrifugation. The supernatant solution was applied to a column of Sephadex G-75 (2.8 x 95 cm), which was eluted with the same buffer. Individual fractions (5.5 ml each) were collected and absorption at 275 nm was determined. The compound emerged from the column as a single component with a slight

tailing. The fractions corresponding to the main peak (Fig. 6-a, tube Nos. 57-80) were combined and the solvent and NH_4HCO_3 were removed by repeated lyophilization to give a fluffy powder (42.5 mg, 54%). The activity against yeast RNA was 12%.

In the preliminary run, starting with the protected RNase (56 mg), the yield of the crude oxidized product was 28.2 mg and the activity was 9%.

Affinity chromatographic purification of the oxidized product.

The oxidized product (40.1 mg) was dissolved in 0.02 M AcONa (pH 5.2) (1 ml) and the solution was applied to a column of Sepharose-(4B)-5'-(4-aminophenylphosphoryl)-uridine-2'-(3')-phosphate (0.8 x 12.3 cm, Vb 6.2 ml), which was eluted first with 0.02 M AcONa, pH 5.2, with which one peak was detected at 275 nm, when individual fractions (3 ml each) were collected. The fractions corresponding to this peak (Fig. 7-a, tube Nos. 3-15) were collected and the solution was lyophilized. The residue was desalted by gel-filtration on Sephadex G-25 with 0.1 N AcOH as eluate. Lyophilization gave a fluffy powder (22.4 mg, inactive). After elution of peak 1, the column was eluted with 0.2 N AcOH. The 2nd peak (Fig. 7-a, tube Nos. 33-40) was detected. Lyophilization of the solvent of peak 2 followed by desalting on Sephadex G-25 afforded a white fluffy powder (5.5 mg, 14%). Yield of this affinity purified product from the protected RNase was 7.4%. Activity against yeast RNA was 82% (85% in 2 $\mu\text{g}/\text{ml}$, 78% in 4 $\mu\text{g}/2\text{ml}$ and 84% in 8 $\mu\text{g}/2\text{ml}$).

Purification of the affinity purified product by ion-exchange chromatography on CM-cellulose.

The affinity purified sample (4.61 mg) was dissolved in a small amount of pH 6.0, 0.01 M sodium phosphate buffer and the solution was applied to a column of CM-cellulose (1 x 11.8 cm), which was

eluted with the same buffer (64 ml) and then by gradient elution with pH 7.5, 0.1 M phosphate buffer (250 ml) through a mixing flask containing the above 0.01 M phosphate buffer (120 ml). Individual fractions (2.0 ml each) were collected and the absorption at 275 nm was determined. Fractions corresponding to the main peak (Fig. 8-a, tube Nos. 59-65), present in the gradient eluates, were combined and the solvent was removed by lyophilization. The residue was desalted by gel-filtration on Sephadex G-25 with 0.1 N AcOH as eluent. After lyophilization, the product was obtained as a white fluffy powder (yield 2.82 mg, 61%).

Amino acid ratios in the 6N HCl (48 hr) hydrolysate are given in Table 6 (Found: C, 41.56; H, 3.74; N, 13.72%). $[\alpha]_D^{23} - 70.8^\circ$ (c 0.7 in 0.1M KCl), (lit.^{117-a}) $- 73.3^\circ$ in 0.1M KCl).

Comparison of synthetic RNase and natural RNase.

1. Chromatographic comparison on CM-cellulose.

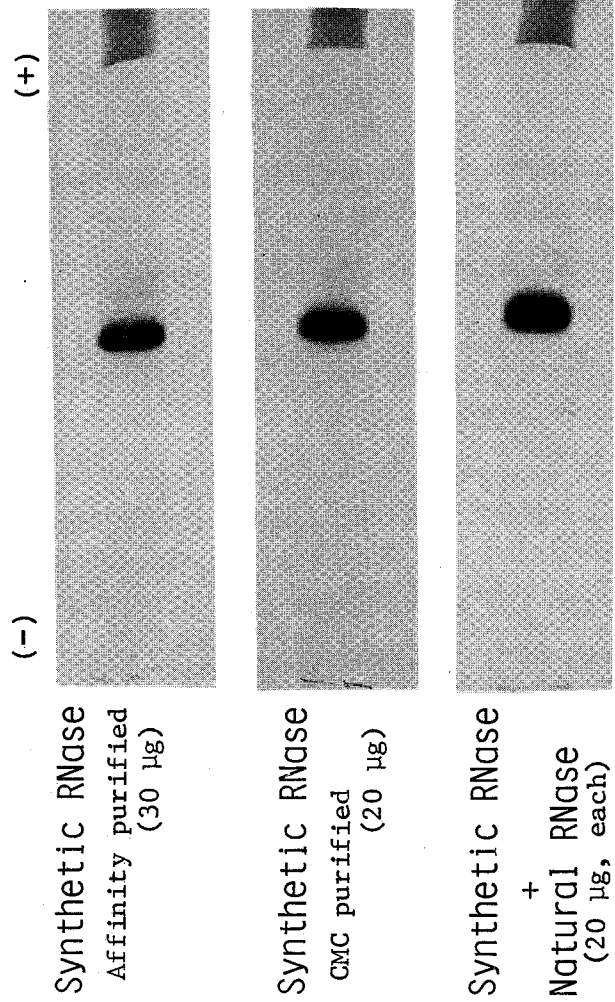
Natural RNase (6.11 mg) was applied to a column of CM-cellulose (1 x 11.8 cm), which was eluted with pH 6.0, 0.01M sodium phosphate buffer and subsequently by gradient elution with pH 7.5, 0.1M phosphate buffer as mentioned in the purification of synthetic RNase. The maximum of the peak coincided with that of synthetic RNase (tube Nos. 62).

2. Disc electrophoretic mobility.

Disc electrophoresis was performed on 15% polyacrylamide gel at pH 4.3 at 2 mA/tube for 2 hr, using methylgreen as a marker. The sample was stained with Coomassie Brilliant Blue R (Sigma). The affinity purified sample (30 μ g) exhibited a main band with a faint impurity, 0.46 and 0.41 x methylgreen respectively, from the origin toward the cathode. The CMC purified sample (20 μ g) behaved as a single component with the mobility of 0.46 x methylgreen from the origin toward the cathode. The identical mobility was confirmed by

Fig. 9

Disc electrophoresis of synthetic RNase A at pH 4.3



disc electrophoresis of a mixing sample of synthetic RNase and natural RNase (20 μ g each) in the same tube (Fig. 9).

3. Ultraviolet spectrum.

The UV absorption spectrum of synthetic RNase (1.771 mg/ml) was compared with that of natural RNase (1.826 mg/ml) in 0.1M KCl solution and the data is shown in Fig. 10. The spectra were superimposable. The maximal molar extinction at 277.5 nm was 9700, when the literature value,^{117-a)} ϵ_{\max} 9800, was taken as a basis of calculation.

4. Michaelis constant.

According to the Kunitz method,^{119-a)} the value was determined by measuring the initial velocities of RNA hydrolysis spectrophotometrically at 300 nm. Sample of synthetic RNase and natural RNase (5 μ g in 2 ml H_2O , each) were mixed with 2 ml of yeast RNA solutions (4.00, 2.00, 1.00, 0.50, 0.40, 0.33, 0.25 mg per ml) in pH 5.0, 0.1M sodium acetate (total volume 4 ml). The absorption at 300 nm was measured for 10 min. (Fig. 11). The Michaelis constants were then calculated from Lineweaver-Burk plots. The K_m values were 1.22 mg/ml and 1.20 mg/ml for synthetic RNase and natural RNase respectively (lit. value,¹²¹⁾ 1.25 mg/ml).

Evaluation of enzymic activity of synthetic RNase.

1. Yeast RNA as substrate.

The Kunitz method^{119-a)} was applied to determine the activity of synthetic RNase at each purification step. Solution containing yeast RNA (2 mg) in pH 5.0, 0.1M sodium acetate buffer (2 ml) were mixed with a solution of synthetic RNase (2 μ g, 4 μ g and 8 μ g each) in H_2O (2 ml each). The reactions were followed spectrophotometrically at 300 nm for 10 min. A solution of natural RNase (2 μ g, 4 μ g and 8 μ g each) in H_2O (2 ml) was allowed to react with yeast RNA as a standard. The specific activities of the synthetic RNase samples

Fig. 10. Ultraviolet spectra of synthetic RNase A

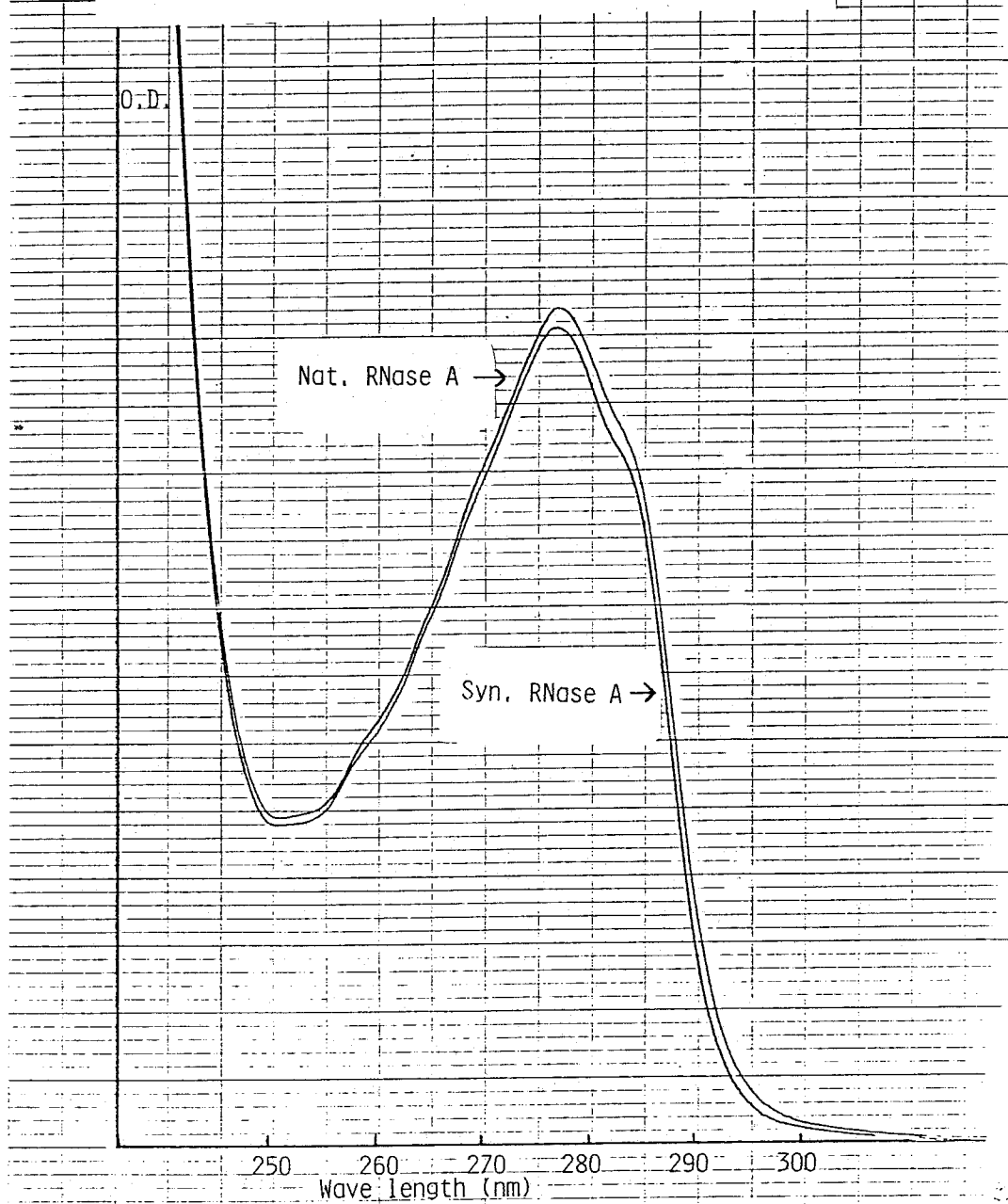


Fig. 11.

Calculation of Michaelis constant (K_m)
for synthetic RNase A

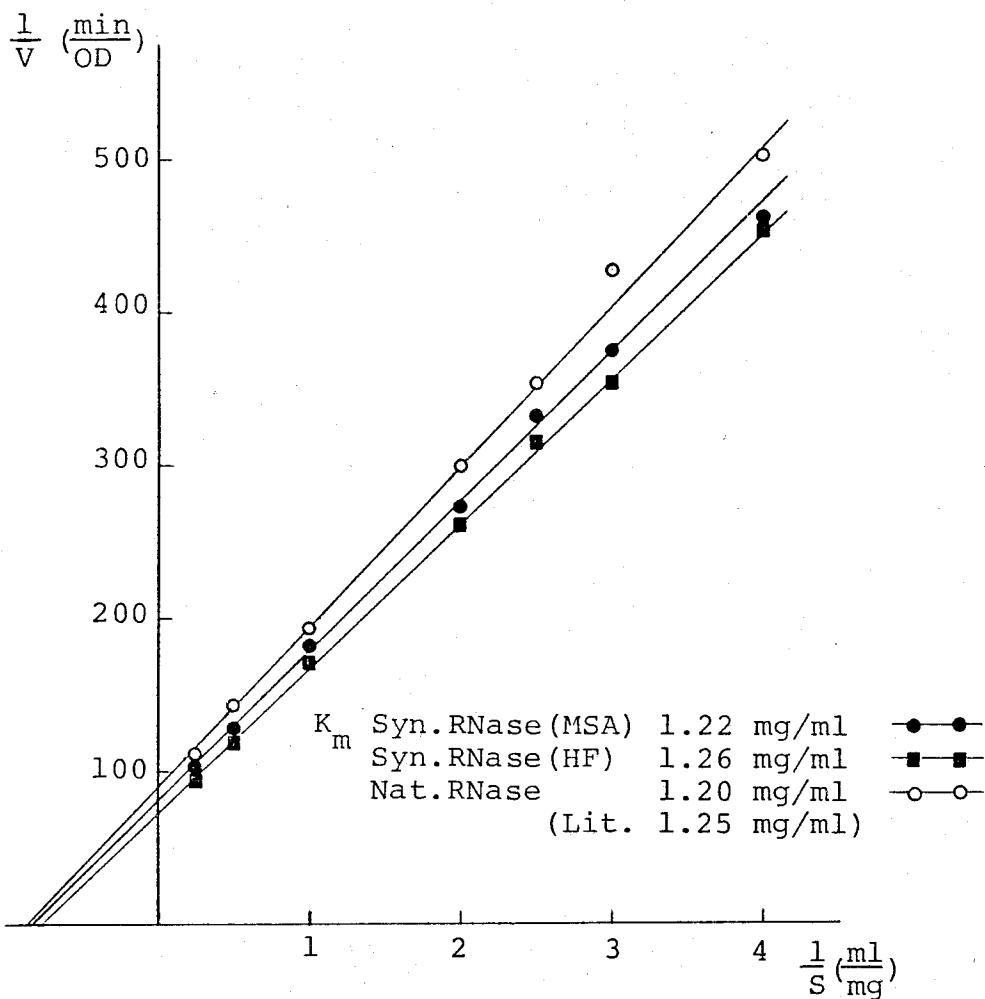
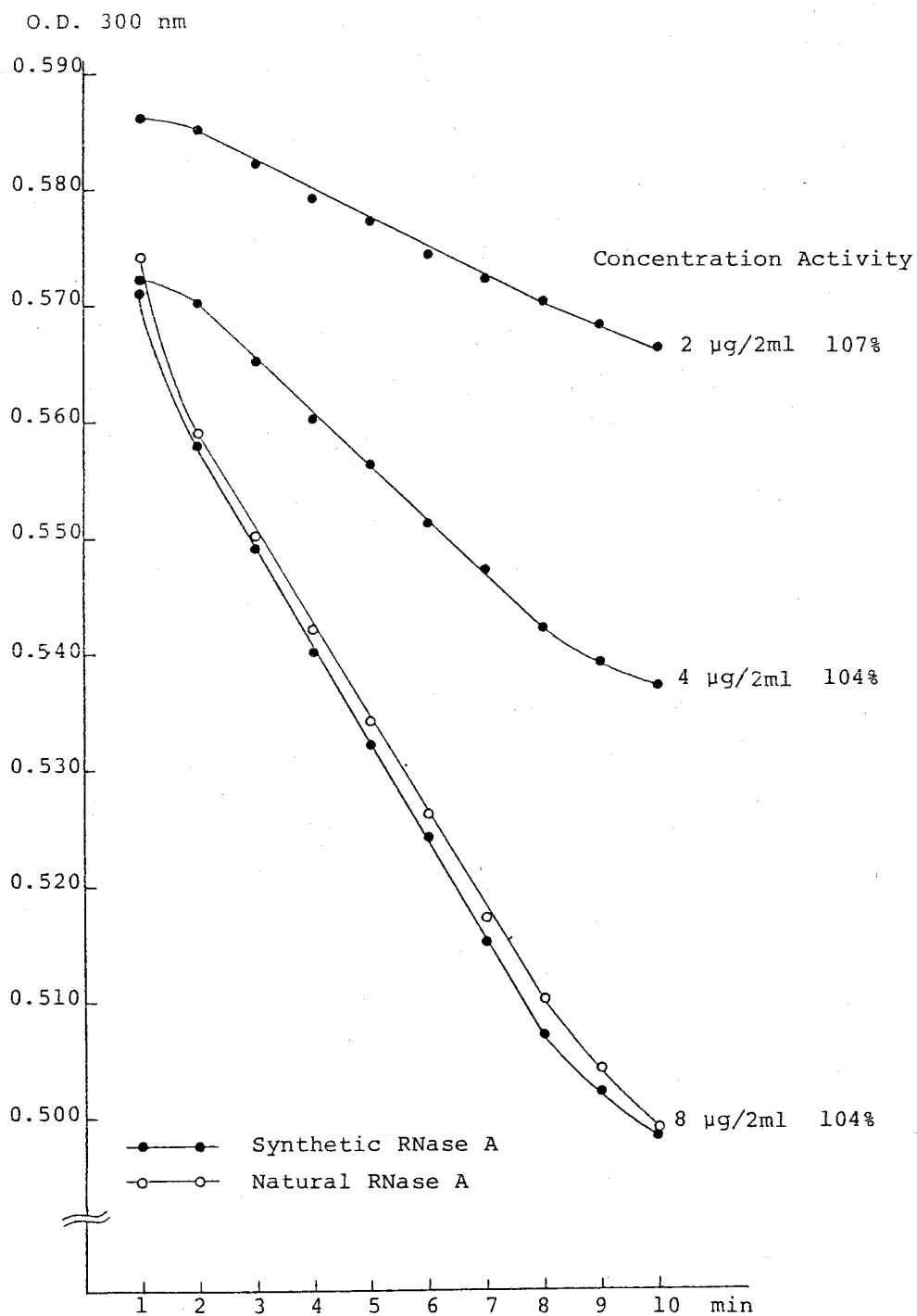


Fig 12. Enzymic activity of synthetic RNase A with yeast RNA as substrate (Kunitz method)



were calculated from the initial slope of the curves. An average activity of the CMC-purified sample (Fig. 12) was 105% (107% in 2 $\mu\text{g}/2\text{ ml}$, 104% in 4 $\mu\text{g}/2\text{ ml}$ and 104% in 8 $\mu\text{g}/2\text{ ml}$).

2. 2',3'-Cyclic cytidine phosphate as substrate.

The fruchter and Crestfield method¹²²⁾ was applied to determine the specific activity of RNase purified by ion-exchange chromatography on CM-cellulose. A solution of synthetic RNase (20 μg) in H_2O (20 μl) was mixed with a solution of 2',3'-cyclic cytidine phosphate (1.4 mg) in 2 ml of pH 7.48, Tris-NaCl buffer (0.012M in Tris, 0.025M in NaCl and 0.01M in HCl). After stirring for 10 min at 0°, 0.2M mercuric chloride (50 μl) was added to stop the reaction. In order to obtain a better separation of 3'-cytidine phosphate from undigested substrate, the reported procedure was modified as follows. The reaction mixture was applied to a column of Amberlite CG-400 (0.9 x 6.7 cm) equilibrated with pH 7.48 Tris-NaCl buffer (0.012M in Tris, 0.025M in NaCl and 0.010M in HCl). The column was then eluted by a linear concentration gradient with pH 7.48 Tris-NaCl buffer (0.060M in Tris, 0.125M in NaCl and 0.050M in HCl) through a mixing flask (200 ml) containing the former buffer at a flow rate of 130 ml/hr. The optical density at 271 nm of the effluents was recorded by an Hitachi wavelength tunable effluent monitor with 10 mm light path flow cell. After elution of undigested substrate, the area of Peak 2 (3'-cytidine phosphate) was compared with that of natural RNase (20 μg) as a standard (Fig. 13). Three parallel experiments were performed and an average activity of 104% was found in synthetic RNase.

3. DNA as substrate.

Referring to the Kunitz method¹²³⁾ for the DNase assay, behaviour of synthetic RNase against calf thymus DNA was examined. A solution of DNA (0.12 mg) in pH 5.0 sodium acetate buffer (3 ml) containing

Fig. 13.

Enzymic activity of synthetic RNase A toward 2',3'-cyclic cytidine phosphate as substrate

Elution: Column Amberlite CG-400 (0.9 x 6.7 cm)
Gradient with Tris-HCl-NaCl buffer (pH 7.48)

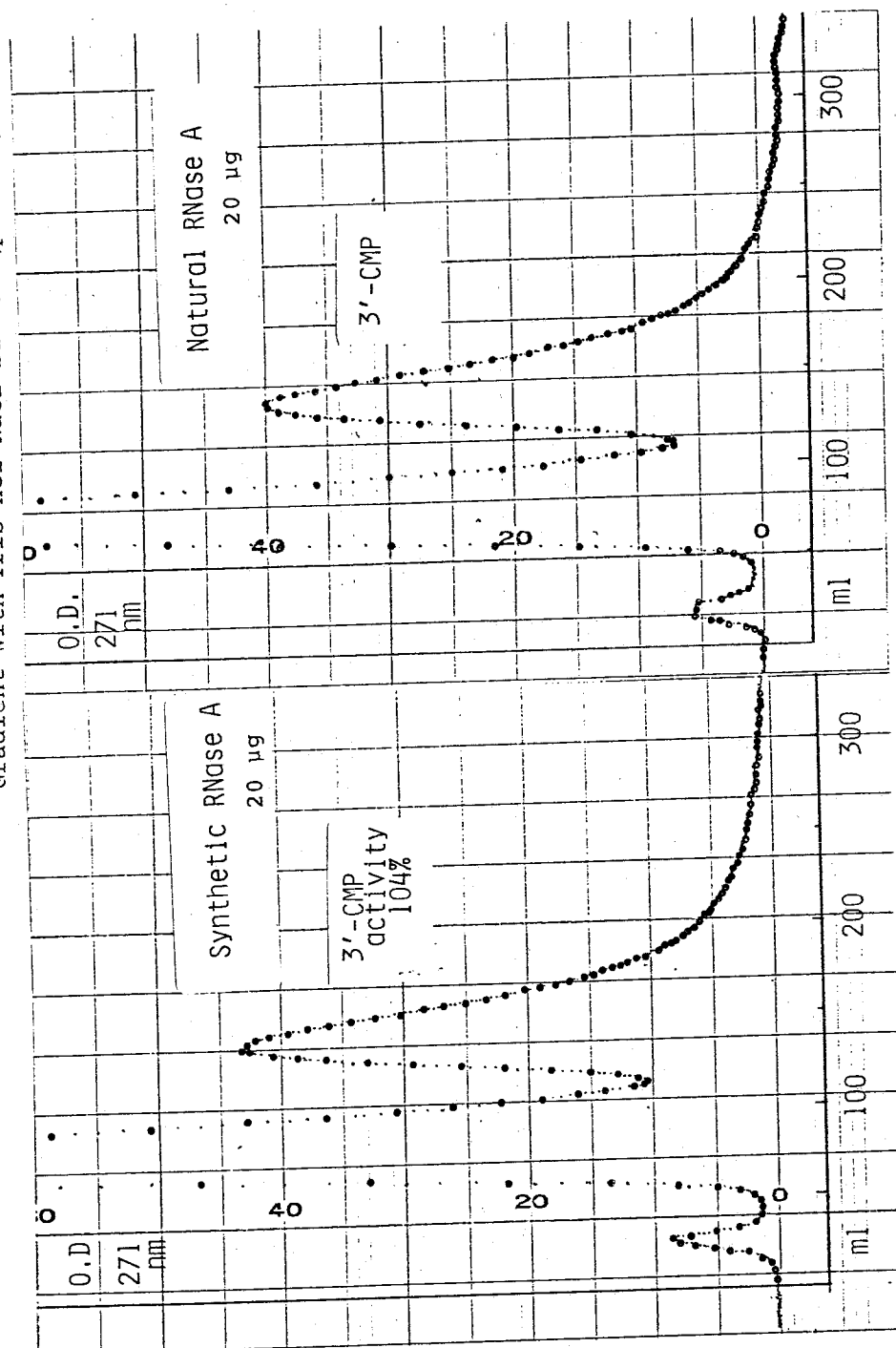
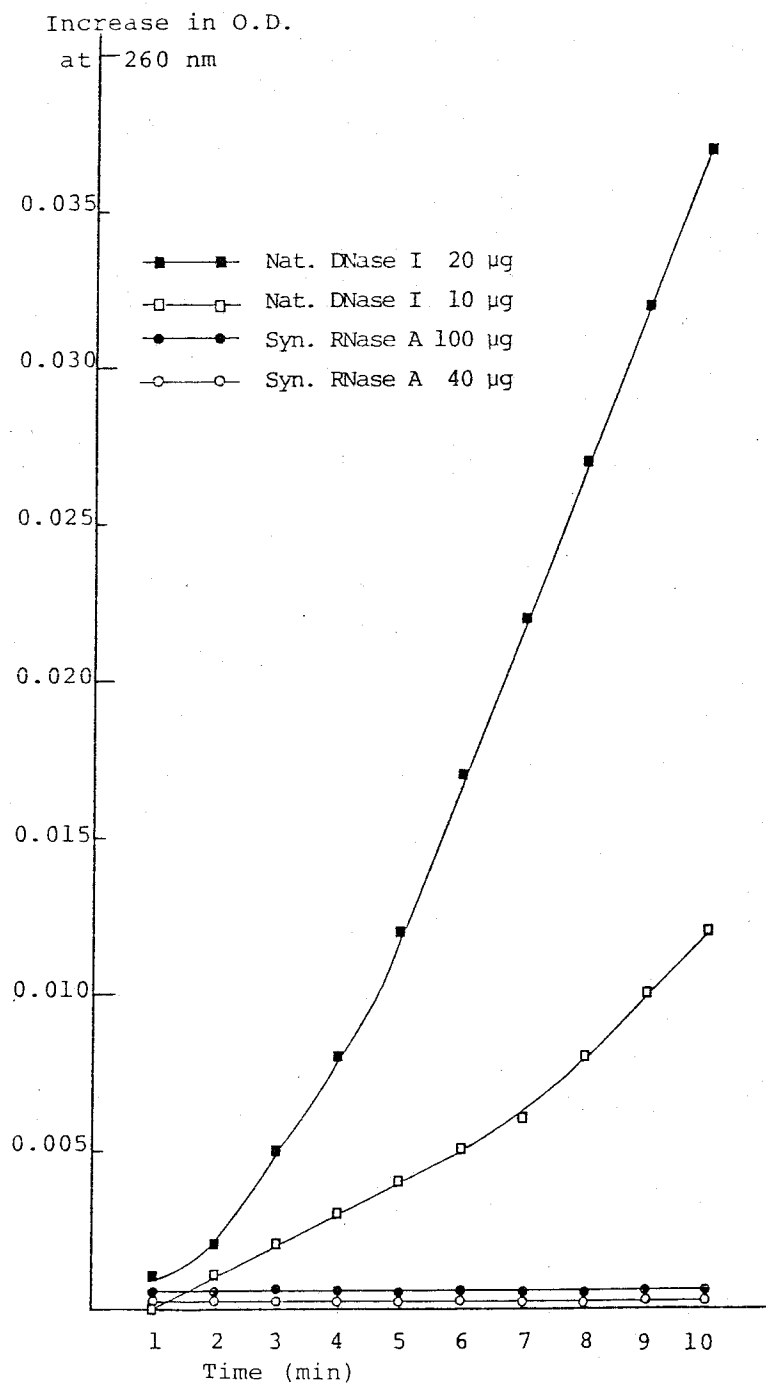


Fig. 14.

DNase activity with synthetic RNase A, using calf thymus DNA
as substrate (Kunitz method)



0.005M MgSO_4 , was mixed with a solution of synthetic RNase (40 μg and 100 μg each) and natural DNase I (10 μg and 20 μg each) in H_2O (1 ml) at 25° . The change of the optical densities was followed spectrophotometrically at 260 nm for 10 min (Fig. 14). The absorption of the solution containing synthetic RNase showed no increase up to 2 hr.

[B] Deprotection of the protected RNase by HF. —————

The protected RNase reduced by thiophenol (100 mg) was treated with HF (approximately 4 ml) in the presence of m-cresol (0.39 ml, 660 equiv.) in an ice-bath for 120 min. Excess HF was removed by evaporation and dry ether was added, the resulting powder was dissolved in 0.2M Tris-HCl buffer (3 ml) containing 4M guanidine HCl. The pH of the solution was adjusted to 8.6 with 5% methylamine. Under an N_2 atmosphere, the solution was incubated in the presence of 2-mercaptoethanol (0.35 ml, 800 equiv.) and dithiothreitol (174 mg, 200 equiv.) at 50° for 24 hr and, after further addition of 2-mercaptoethanol (0.18 ml, 400 equiv.), at room temperature for 40 hr, as described in the MSA deprotection. After adjusting the pH to 3 with 3N HCl, the solution was applied to a column of Sephadex G-25 (1.6 x 68 cm) with 0.1N AcOH as eluate for desalting. Individual fractions (3 ml each) were collected.

Air oxidation of the HF deprotected product. —————

Fractions corresponding to the front peak (tube Nos. 18-34) were combined and the solution was diluted with H_2O (3 l). The protein concentration was brought to 0.02 mg/ml with pH 8.2, 0.2M Tris-HCl buffer (1 l). After stirring for 15 min, the solution was kept standing at room temperature for 2 days, while the Ellman color intensity dropped from 0.118 to the constant value, 0.004. The pH of the solution was adjusted to 3 with 2N HCl and lyophilized. The residue was dialyzed three times against distilled H_2O (1 l) using

cellulose tubing (VT 351) . The content of the tube was lyophilized to give a hygroscopic powder (172 mg). The crude product was dissolved in a small amount of 0.05M NH_4HCO_3 and insoluble material was removed by centrifugation (8 mg). The supernatant was applied to a column of Sephadex G-75 (2.8 x 95 cm, Vb 585 ml) with 0.05M NH_4HCO_3 as eluate as mentioned in the MSA deprotection. Fractions corresponding to the main peak (Fig. 6-b, tube Nos. 57-80) were combined and the solvent was removed by lyophilization to give a fluffy powder; yield 47.1 mg (60%). The activity against yeast RNA was 17%.

Purification of the air oxidized product by affinity chromatography.

The crude air oxidized product (44.12 mg) was dissolved in a small amount of 0.02N AcONa and the solution was applied to an affinity column (2.2 x 8.6 cm), and was eluted with the same buffer (150 ml) and then 0.2N AcOH (100 ml) as mentioned in the MSA deprotection. Peak 1 (Fig. 7-b, tube Nos. 19-40) in 0.02M AcONa eluates gave, after desalting, an inactive material (26.13 mg). Peak 2 (tube Nos. 57-74) in 0.2N AcOH eluates gave an active material (4.79 mg, yield 11%). The activity against yeast RNA was 82% (79% in 2 $\mu\text{g}/2$ ml, 82% in 4 $\mu\text{g}/2$ ml and 84% in 8 $\mu\text{g}/2$ ml).

Purification of the affinity purified product by ion-exchange chromatography on CM-cellulose.

The affinity purified sample (3.99 mg) was applied to a column of CM-cellulose (1 x 11.8 cm), which was eluted with 0.01M sodium phosphate buffer (pH 6.0) (64 ml) and then by gradient elution with 0.1M phosphate buffer (pH 7.5) through a mixing flask containing the above 0.01M buffer (120 ml). Fractions corresponding to the main peak (Fig. 8-b, tube Nos. 59-65) were combined and the solvent was removed by lyophilization. The residue was desalted by gel-

filtration on Sephadex G-25 with 0.1N AcOH as eluate. After lyophilization, a white fluffy powder was obtained; 3.02 mg (76%). Total yield 5%. The activity against yeast RNA was 106% (108% in 2 μ g/2 ml, 107% in 4 μ g/2 ml and 103% in 8 μ g/2 ml). Michaelis constant, K_m 1.26 mg/ml. Amino acid ratios in the 6N HCl (48 hr) hydrolysate (numbers in parentheses are theory): Asp 14.74(15), Thr 9.65(10), Ser 13.84(15), Glu 12.49(12), Pro 4.45(4), Gly 3.31(3), Ala 12.47(12), Cys 3.70(4), Val 8.67(9), Met 3.71(4), Ile 2.38(3), Leu 2.00(2), Tyr 5.96(6), Phe 3.07(3), Lys 10.21(10), His 3.59(4), Arg 3.70(4), recovery of standard Leu 79%.

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